Attorney's Docket No. 5800-2B (35800/190593) PATENT

AF #1635

In The United States Patent And Trademark Office

#17

In re: Glucksmann *et al.* Group Art Unit: 1635
Appl. No.: 09/464,685 Examiner: A. Wang
Filed: December 16, 1999
For: 2871 RECEPTOR, A NOVEL G-PROTEIN COUPLED RECEPTOR

July 10, 2001

Commissioner for Patents
Washington, DC 20231

APPEAL BRIEF TRANSMITTAL
(PATENT APPLICATION – 37 C.F.R. § 1.192)

1. Transmitted herewith, in triplicate, is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on May 11, 2001.

2. This application is filed on behalf of
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3. Pursuant to 37 C.F.R. § 1.17(c), the fee for filing the Appeal Brief is:
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CERTIFICATE OF MAILING

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Attorney's Docket No. 5800-2B (35800/190593) PATENT

Appeal Brief

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Glucksmann *et al.*		
Appl. No.:	09/464,685	Group Art Unit:	1635
Filed:	December 16, 1999	Examiner:	A. Wang
For:	2871 RECEPTOR, A NOVEL G-PROTEIN COUPLED RECEPTOR		

July 10, 2001

Commissioner for Patents
Washington, DC 20231

APPEAL BRIEF

Sir:

This Appeal Brief is filed pursuant to the "Notice of Appeal to the Board of Patent Appeals and Interferences" filed May 11, 2001.

Real Party in Interest.

The real party in interest in this appeal is Millennium Pharmaceuticals, Inc., the assignee of the above-referenced patent application.

Related Appeals and Interferences.

An appeal brief is concurrently being filed in Application Serial No. 09/324,465. The present application is a continuation-in-part application of co-pending Application Serial No. 09/324,465.

Status of Claims.

Claims 73, 74, 81, and 88-96 are the subject of this appeal. The claims appear in Appendix A. Claims 1-72, 75-80, 82-87, and 97-103 have been cancelled.

Status of Amendments.

A Supplemental Amendment was filed July 6, 2001 to cancel claims 60-72, 75-80, 82-87, and 97-103 as these claims were withdrawn subject to the restriction requirement.

Summary of the Invention.

The pending claims of the present invention are directed to methods for detecting the presence of a polypeptide wherein the polypeptide is the G-protein coupled receptor (GPCR) 2871 and fragments and variants of 2871, methods for modulating the activity of a polypeptide wherein the polypeptide is the GPCR 2871 and fragments and variants of 2871, methods for screening a cell to identify agents that bind the 2871 GPCR polypeptide, methods for screening a cell to identify an agent that modulates the expression level or activity of the 2871 GPCR polypeptide, and a method for assessing expression of the 2871 GPCR in disease states in a patient. The 2871 receptor shares a high level of sequence similarity with a rhodopsin family GPCR consensus domain and contains a GPCR signature sequence. The 2871 receptor is a member of a family of proteins that are known in the art for their importance as therapeutic targets.

Issues.

Issue 1--Whether the inventions of claims 73, 74, 81, and 88-96 have utility under 35 U.S.C. § 101 and are thus enabled under 35 U.S.C. § 112, first paragraph.

While the Examiner has rejected the claims under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, both rejections hinge on whether Applicants have established that the 2871 polypeptide is a GPCR. The Examiner states, "[a]lthough Applicants do indeed provide multiple well established and specific utilities for a GPCR, Applicants have not clearly demonstrated that the cloned nucleic acid and its encoded polypeptide is actually a GPCR as was noted in the utility rejection." (February 12, 2001 Office Action, page 3). Therefore both rejections are based on the fact that the Examiner has not accepted the evidence that the 2871 polypeptide is a GPCR.

Grouping of the Claims.

The claims do stand or fall together. The rejection of all the claims hinges on the fact that the Examiner does not accept Applicants' arguments that the 2871 polypeptide is a GPCR. For this reason, all the claims stand or fall together.

Argument.

Issue 1--Whether the invention of claims 73, 74, 81, and 88-96 has utility under 35 U.S.C. § 101 and thus is enabled under 35 U.S.C. § 112, first paragraph.

The Examiner has rejected claims 73, 74, 81, and 88-96 under 35 U.S.C. § 101 as lacking patentable utility and as lacking enablement under 35 U.S.C. § 112, first paragraph. The Examiner indicates that "Applicants have not clearly demonstrated that the cloned nucleic acid and its encoded polypeptide is actually a GPCR" (February 12, 2001 Office Action, page 3). In fact, Applicants have provided art-accepted methods to establish that the 2871 polypeptide is a GPCR as outlined in detail below.

I. 2871 Encodes a G-Protein Coupled Receptor.

A. Analysis of the 2871 sequence demonstrates that 2871 is a G-protein coupled receptor.

1. 2871 contains a Pfam seven transmembrane receptor domain for the rhodopsin family of GPCRs.

The 2871 polypeptide has been compared to the Pfam database of protein families and been shown to share a high degree of sequence similarity with the consensus domain for the seven transmembrane receptor domain consensus sequence for the rhodopsin family of G-protein coupled receptors (PFAM Accession No. PF00001; see Figure 2). The Pfam database provides a curated collection of well-characterized protein family domains with high quality alignments. Functional domains of novel proteins may be identified by comparison with the Pfam protein family domain alignments. It is well known in the art that regions of sequence homology with known functional domains may be used to determine protein function. Accordingly, the presence of a Pfam seven transmembrane receptor domain for the rhodopsin family of GPCRs in the 2871 sequence indicates that 2871 functions as a G-protein coupled receptor.

2. 2871 contains a Prosite G-protein coupled receptor signature sequence and a conserved GPCR signal transduction signature.

The 2871 signature sequence includes the highly-conserved GPCR signal transduction signature "DRY" (amino acids 138-140 of SEQ ID NO:1); this consensus signature is believed to be involved in G-protein interaction. See page 22, lines 16-17 of the specification. Accordingly, 2871 contains conserved sequences required for GPCR function.

3. The evidence presented in the specification provides strong support for the conclusion that 2871 functions as a G-protein coupled receptor.

The Examiner in the present case has not accepted Applicants' assertion that 2871 functions as a G-protein coupled receptor. However, page 22 of the specification sets forth an analysis of the 2871 polypeptide. See, particularly lines 12-17, where it is stated that "[t]he transmembrane domain includes a GPCR signal transduction signature, DRY, at residues 138-140." Attention is also drawn to Figures 2 and 3 as well as the description of the figures, page 5 of the specification. Figure 2 indicates the presence of a Pfam seven transmembrane receptor domain for the rhodopsin family of GPCRs in the 2871 sequence, which supports Applicants' position that 2871 functions as a GPCR. Multiple lines of evidence overwhelmingly support the conclusion that 2871 functions as a G-protein coupled receptor, as described above. In view of the evidence provided, one of skill in the art would readily accept Applicants' asserted utility for 2871.

B. Sequence homology is an acceptable basis for utility according to United States Patent and Trademark Office Utility Examination Guidelines.

1. The utility of the invention set forth in claims 73, 74, 81, and 88-96 meets the standard set by the Utility Examination Guidelines.

The United States Patent and Trademark Office "Utility Examination Guidelines" (66 Fed Reg. 1092 (2001)) make it clear that sequence homology is sufficient to establish utility, and that, contrary to the utility standard set by the Examiner, working examples or biochemical evidence

are not a *per se* requirement for the establishment of utility. The "Utility Examination Guidelines" state, "[w]hen a patent application claiming a nucleic acid asserts a specific, substantial, and credible utility, and bases the assertion upon homology to existing nucleic acids or proteins having an accepted utility, the asserted utility must be accepted by the examiner unless the Office has sufficient evidence or sound scientific reasoning to rebut such an assertion" (66 Fed. Reg. 1096). In the present case, the Examiner has not accepted the asserted utility for the claimed invention but has failed to provide sufficient evidence or sound scientific reasoning to rebut Applicants' assertions.

2. The asserted utility for claims 73, 74, 81, and 88-96 meets the utility standard as exemplified in the Revised Interim Utility Guidelines Training Material Examples.

The "Revised Interim Utility Guidelines Training Material Examples" (http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf; December 21, 1999) are not correctly applied in the rejection of claims 73, 74, 81, and 88-96. Of the examples given in the training materials, Example 10, which is directed to a sequence that has sequence similarity with a DNA ligase, is most analogous to the present application. As in Example 10, the 2871 receptor has been shown to share sequence similarity with a protein family of known function. As in Example 10, the protein family of known function has a well-established utility, as described more fully below. Accordingly, based on analogy to Example 10, the present invention also meets the criteria for well-established utility.

The sequence of Example 10 is accorded to have a specific and substantial utility according to the "Revised Interim Utility Guidelines Training Material Examples," *ibid.*, despite the fact that the encoded polypeptide has not been directly demonstrated to have DNA ligase activity, and the substrate (i.e., single-stranded DNA or double-stranded DNA, blunt-ended DNA, 5' recessed ended DNA, 3' recessed ended DNA), co-factor requirements, and reaction conditions required to practice the invention are not disclosed. Thus, in accordance with the Utility Examination Guidelines, the very fact that the sequence of Example 10 has sequence similarity with a known protein possessing well-established utility is sufficient to confer a specific, substantial, and credible utility upon the claimed sequence. As the claimed invention of

the present application is analogous to the situation described in Example 10, the criteria for utility have been met for claims 73, 74, 81, and 88-96.

3. The rejection of claims 73, 74, 81, and 88-96 under 35 U.S.C. § 101 is inconsistent with the USPTO Utility Examination Guidelines.

In rejecting Applicants' asserted utility for the 2871 receptor, the Examiner states, "that a protein's activity cannot be predicted based on primary structure alone" (August 25, 2000 Office Action, pages 4-5). However, the rejection of the claims under U.S.C. § 101 on these grounds is inconsistent with the current USPTO "Utility Examination Guidelines" as demonstrated above. The invention of claims 73, 74, 81, and 88-96 meets the standard set forth in the guidelines, and thus reversal of the rejection under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, is respectfully requested.

C. Those of skill in the art recognize that sequence homology may be used to determine protein function.

1. Experimental evidence supports the use of domain sequence-based predictions of protein function.

The prior art is replete with examples of proteins whose functions have been determined based on sequence similarity with proteins or domains of known function and later confirmed using a biochemical or genetic approach. One such example is seen in Nguyen *et al.* (2001) *Mol. Pharmacol.* 59:427-433, a copy of which is provided herewith as Appendix B. This reference describes the identification of the H4 receptor based on a query of GenBank to identify sequences sharing sequence similarity with GPCRs (see page 428, column 1, first full paragraph). The H4 polypeptide was predicted, based on sequence similarity with the histamine receptor H3, to have histamine receptor function. Subsequently, biochemical assays confirmed that histamine serves as a ligand for the H4 receptor and causes the receptor's internalization.

Another example is described in Dickman (1997) *Science 277*:1605-1606, a copy of which is provided herewith as Appendix C. This reference describes the cloning and

characterization of p73, a homolog of the tumor suppressor protein p53. The p73 polypeptide was predicted to have tumor suppressor activity based on its sequence similarity with p53 in several key domains, including the transcription activation domain, DNA binding domain, and oligomerization domain (see page 160, column 3, first full paragraph). In accordance with this, p73 has subsequently been shown to have many of the activities of p53, including the ability to initiate cell death using the same pathway utilized by p53. Accordingly, sequence similarity to p53 was shown to be an accurate predictor of p73 activity.

Yet another example is described in Kliewer *et al.* (1998) *Cell 92*:73-82, a copy of which is provided herewith as Appendix D. This reference describes the identification of two novel nuclear receptors based on sequence similarity with the ligand binding domains of known nuclear receptors (see page 74, column 1, first full paragraph). The novel receptors, termed pregnane X receptor 1 and 2 (PXR.1 and PXR.2) were predicted to have nuclear receptor activity, i.e., hormone-regulated transcriptional activity, based on this sequence similarity. Biochemical data presented in the reference indicates that PXR regulates the transcription of the CYP3A family of steroid hydroxylases in a pregnane-dependent manner, confirming the functional determination based on DNA binding domain and ligand binding domain sequence similarity.

The examples given here, which demonstrate the accuracy of sequence similarity-based determinations of protein function, represent only a few of the many such instances that are found in the scientific literature. Because of evidence such as that presented above, methods of using sequence homology within the functional domains of proteins of known function to determine the function of novel proteins have become widely accepted by those of skill in the art as reliable and accurate. Accordingly, one of skill in the art would readily accept that 2871 functions as a GPCR based on the types evidence presented in specification.

2. The Examiner has not presented sufficient evidence or reasoning to rebut the Applicants' asserted utility.

The USPTO "Utility Examination Guidelines" state, "[a] patent examiner must accept a utility asserted by Applicants unless the Office has evidence or sound scientific reasoning to rebut the assertion. The examiner's decision must be supported by a preponderance of all the

evidence on record" (66 Fed. Reg.1096). In rejecting claims 73, 74, 81, and 88-96 under 35 U.S.C. § 101, the Examiner in the present case states that sequence-to-function methods of assigning function are prone to errors and cites Berendson (1998) *Science 282*:642-643 and Galperin *et al.* (2000) *Nature Biotech. 18*:609-613 in support of this statement. However, the findings of these references are not applicable to the particular types of sequence comparison data presented in the present application.

Berendson's comments that the Examiner cites are directed toward predicting the native conformation of a protein of known amino acid sequence, not towards predicting merely activity. Berendson notes that "the obvious route to that goal [the quest for the structure and fix of the coded proteins] is by homology modeling: use as much information as you can get from the database of known structures." As discussed elsewhere herein, the GPCR family of proteins is well-characterized and described.

Galperin *et al.* report that predictions of function based on sequence identity can be problematic under certain conditions. This reference addresses functional predictions based on sequence comparisons when a protein has no homologs in current databases or when all database hits are to uncharacterized gene products. (Galperin *et al.*, Supra, page 609, column 1, paragraph 2).

The function of the 2871 receptor has not been determined based on sequence identity to such a protein family. Rather, the function of this receptor has been determined based on sequence homology with G-protein coupled receptors, including a high degree of sequence similarity to the Pfam seven transmembrane receptor domain consensus sequence for the rhodopsin family of GPCRs and the presence of a Prosite G-protein coupled receptor signature. The proteins included in the Pfam seed alignment for the seven transmembrane receptor domain consensus sequence include numerous GPCRs that have been well-characterized biochemically, for example, the serotonin receptors $5HT_{1A}$, $5HT_{1B}$, and 5HT-7, the β-1 and α-2 adrenergic receptors, the dopamine receptor, and the muscarinic acetylcholine receptor M1 (see Appendix E), clearly distinguishing this family from the uncharacterized gene products mentioned by Galperin *et al.*

3. The scientific evidence supports the validity of the methods used to determine 2871 function.

The Examiner has failed to accept Applicants' asserted utility for the 2871 polypeptide on the grounds that it is based on sequence similarity between 2871 and G-protein coupled receptors. The final Office Action states, "the mere presence of said domains [which confer protein activity] do not adequately conform or define specifications to an isolated polypeptide, particularly to GPCR's" (December 4, 2000 Office Action, page 4). However, the rejection is inconsistent with the view of those of skill in the art as applied to the facts of the present case. Those of skill in the art know that the use of sequence similarity, particularly sequence similarity within functional domains, is a widely-accepted, reliable tool for determining protein function, as demonstrated above. Therefore, absent evidence to the contrary, Applicants' asserted utility must be accepted.

II. Conclusion

Applicants have demonstrated that 2871 functions as a G-protein coupled receptor, as evidenced by the fact that the 2871 polypeptide contains a rhodopsin family G-protein coupled receptor consensus domain and a G-protein coupled receptor signature sequence. The methods used to determine the function of 2871 are supported by experimental evidence and accepted by those of skill in the art. As the Examiner has acknowledged (February 12, 2001 Office Action, page 3) that "applicants do indeed provide multiple well established and specific utilities for a GPCR," having established that the 2871 polypeptide is a GPCR, the rejections should be withdrawn.

The Examiner further states that "since the invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above [in the rejection under 35 U.S.C. § 101], one skilled in the art would not know how to use the claimed invention." Office Action of February 12, 2001, page 4. Thus, having established that the 2871 polypeptide is a GPCR, the Examiner's concerns regarding enablement of the claims have been addressed. For all these reasons, the rejections of the claims under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, should be reversed.

CONCLUSION

In view of the arguments presented above, Applicants contend that each of claims 73, 74, 81, and 88-96 is patentable. Therefore, reversal of the rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, is respectfully solicited.

Respectfully submitted,

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Polly P. Burton

Polly P. Burton

In re: Glucksmann *et al.*
Appl. No. 09/464,685
Filed: December 16, 1999

APPENDIX A

APPEALED CLAIMS

73. A method for detecting the presence of a polypeptide having an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO:1;

(b) the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(c) the amino acid sequence of an allelic variant of the amino acid sequence shown in SEQ ID NO:1;

(d) the amino acid sequence of an allelic variant of the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(e) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(f) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369; wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid comprising SEQ ID NO:2, or a complement thereof under stringent conditions; and

(g) a polypeptide which is encoded by a nucleic acid molecule comprising a nucleotide sequence which is at least 45% identical to a nucleic acid comprising the nucleotide sequence of SEQ ID NO:2, or a complement thereof;

said method comprising contacting the sample with a compound which selectively binds to any one of the polypeptides of (a) – (g) and determining whether the compound binds to said polypeptides in the sample.

74. The method of claim 73, wherein the compound which binds to the polypeptide is an antibody.

81. A method for modulating the activity of a polypeptide having an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO:1;

(b) the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(c) the amino acid sequence of an allelic variant of the amino acid sequence shown in SEQ ID NO:1;

(d) the amino acid sequence of an allelic variant of the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(e) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369;

(f) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:1, or an amino acid sequence encoded by the cDNA contained in ATCC Deposit No. PTA-2369; wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid comprising SEQ ID NO:2, or a complement thereof under stringent conditions; and

(g) a polypeptide which is encoded by a nucleic acid molecule comprising a nucleotide sequence which is at least 45% identical to a nucleic acid comprising the nucleotide sequence of SEQ ID NO:2, or a complement thereof;

said method comprising contacting any one of polypeptides (a) – (g) or a cell expressing any one of polypeptides (a) – (g) with a compound which binds to the polypeptide in a sufficient concentration to modulate the activity of the polypeptides.

88. A method for screening a cell to identify an agent that binds with a polypeptide having an amino acid sequence shown in SEQ ID NO:1 in said cell, said method comprising contacting said cell with an agent and detecting an interaction between said polypeptide and agent.

89. A method for screening a cell to identify an agent that modulates the expression level or activity of the polypeptide having an amino acid sequence shown in SEQ ID NO:1 in said cell, said method comprising contacting said cell with an agent and detecting an interaction between said polypeptide and agent.

90. The method of claim 89, wherein said cell is a blood cell.

91. The method of claim 90, wherein said blood cell is a myeloid progenitor cell.

92. The method of claim 91, wherein said myeloid progenitor cell is a $CD34^+$ cell.

93. The method of claim 89, wherein said agent increases the level or activity of said polypeptide.

94. The method of claim 89, wherein said agent decreases the level or activity of said polypeptide.

95. A method for assessing G-protein receptor expression in disease states of a patient, comprising contacting a tissue of said patient with an isolated antibody that selectively binds to the polypeptide shown in SEQ ID NO:1.

96. The method of claim 95, wherein the G-protein coupled receptor expression is involved in signal transduction.

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APPENDIX B

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ACCELERATED COMMUNICATION

Discovery of a Novel Member of the Histamine Receptor Family

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ABSTRACT

We report the discovery, tissue distribution and pharmacological characterization of a novel receptor, which we have named H4. Like the three histamine receptors reported previously (H1, H2, and H3), the H4 receptor is a G protein-coupled receptor and is most closely related to the H3 receptor, sharing 58% identity in the transmembrane regions. The gene encoding the H4 receptor was discovered initially in a search of the GenBank databases as sequence fragments retrieved in a partially sequenced human genomic contig mapped to chromosome 18. These sequences were used to retrieve a partial cDNA clone and, in combination with genomic fragments, were used to determine the full-length open reading frame of 390 amino acids. Northern analysis revealed a 3.0-kb transcript in rat testis and intestine. Radioligand binding studies indicated that the H4 receptor has a unique pharmacology and binds [^{3}H]histamine (K_d = 44 nM) and [^{3}H]pyrilamine (K_d = 32 nM) and several psychoactive compounds (amitriptyline, chlorpromazine, cyproheptadine, mianserin) with moderate affinity (K_i range of 33–750 nM). Additionally, histamine induced a rapid internalization of HA-tagged H4 receptors in transfected human embryonic kidney 293 cells.

Histamine is a monoamine neurotransmitter thus far known to activate three G protein-coupled receptors (GPCRs), the H1, H2, and H3 receptors (Hill et al., 1997). Although molecular cloning has made possible the identification, isolation, and characterization of the majority of known GPCRs, the histamine receptor subtypes have proven more difficult to identify. The first two histamine receptor genes cloned were H1 (Yamashita et al., 1991) and H2 (Gantz et al., 1991). The identification of the H3 receptor came nearly a decade later (Lovenberg et al., 1999). Collectively, the H1, H2, and H3 receptors share less than 35% identity with one another and each has greater sequence identities with other aminergic receptors. Thus, the histamine receptor gene family is significantly divergent and may have evolved from different ancestral genes (Leurs et al., 2000). In addition, there is evidence that multiple subtypes of the H3 receptor may exist. Pharmacological studies performed on membranes extracted from rat brain tissue revealed two classes of H3 binding sites (West et al., 1990; Leurs et al., 1996). However, efforts to clone a second H3 receptor subtype in the brain have thus far been unsuccessful.

Through molecular cloning techniques, we have identified numerous novel GPCRs, including many subtypes not suspected to exist on the basis of pharmacology. Over the past decade, our cloning efforts have identified a number of GPCR genes, including genes encoding such receptors as the cysteinyl leukotriene CysLT2 (Heise et al., 2000), galanin GalR2 and GalR3 (Kolakowski et al., 1998), thyrotropin-releasing hormone TRH-R2 (O'Dowd et al., 2000), uridine nucleotide receptor UNR (Nguyen et al., 1995), as well as a large cohort of orphan GPCRs for which the endogenous ligands remain to be elucidated (Marchese et al., 1999; Lee et al., 2001). In a

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T.N. and D.A.S. contributed equally to this work.

ABBREVIATIONS: GPCR(s), G protein-coupled receptor(s); HTGS, high-throughput genomic sequence; TM, transmembrane; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; HA, hemagglutinin; HEK, human embryonic kidney cells; kb, kilobase pair.

scan of the GenBank high-throughput genomic sequence (HTGS) database, we identified a small DNA fragment that had greatest homology to the known histamine receptor genes. Here we report the discovery, tissue distribution, and pharmacological characterization of human DNA encoding a receptor, H4. H4 receptor mRNA had discrete and limited expression in rat testis and intestine. Although the H4 receptor was most closely related to the H3 receptor [58% identity in the transmembrane (TM) regions], it seemed to possess a unique pharmacology, with highest affinity for psychoactive drugs (amitriptyline, chlorpromazine) with a tricyclic structure.

Materials and Methods

Database Searching. A customized search was used to query the GenBank HTGS database of high-throughput genomic sequences maintained by the National Center for Biotechnology Information (NCBI) with the sequences of various GPCRs, using the FAST_PAN program (Retief et al., 1999).

Screening and Construction of the H4 Receptor Gene. Partial sequences encoding the H4 receptor were used to design primers to PCR-amplify H4-encoding fragments from human genomic DNA. A fragment encoding the start methionine to TM2 was amplified using primers P1 (5′-ATGCCAGATACTAATAGCACAATC-3′) and P2 (5′-CACAAAGAAGTCAGAGATGG-3′) and another fragment encoding from TM5 to TM6 using primers P3 (5′-TGGTACATCCTTGCCATC-3′) and P4 (5′-TATGGAGCCCAGCAAACAG-3′). PCR products were extracted with phenol/chloroform, precipitated with ethanol, and electrophoresed on a low-melting agarose gel. DNA in the expected size range were excised from the gel, ligated into the *Eco*RV site of the pBluescript vector (Stratagene, La Jolla, CA), and then the sequence was determined. H4 receptor-encoding fragments were used to screen human and rat genomic libraries (CLONTECH, Palo Alto, CA) and a human testis cDNA library (CLONTECH), as described previously (Marchese et al., 1994). Isolated phages from the human and rat genomic libraries were purified and subcloned, and the sequence was determined as described previously (Marchese et al., 1994). Isolated phages from the human cDNA library were subjected to PCR amplification using primers specific for regions flanking the insert of the λgt10 library vector, subcloned, and sequenced.

A DNA fragment encoding the full-length human H4 receptor was amplified by PCR in three stages using isolated human genomic and cDNA library phage as templates. In stage 1, three overlapping fragments (A, B, and C), together encoding the full-length H4 receptor, were PCR-amplified as follows. Fragment A was obtained using primers P5 (5′-CATCATTTGATGTGATGCCA-3′) and P6 (5′-CAAAGGAATGGAGATCACACCCACAAAGAAGTCAGA-3′) from an isolated genomic library phage obtained with the Met-TM2 encoding DNA probe. Fragment B was obtained using primers P7 (5′-GTGATCTCCATTCCTTTG-3′) and P8 (5′-TCCAATAAATATTCAT-3′) from an isolated testis cDNA library phage. Fragment C was obtained using primers P9 (5′-TGGTACATCCTTGCCATC-3′) and P10 (5′-GAGGTGAGAAAATTGTC-3′) from an isolated genomic library phage obtained with the TM5–6 encoding DNA probe. In stage 2, fragments B and C were joined by PCR using primers P7 and P10. In stage 3, fragments A and B/C were joined together using primer P5 and P10 to obtain a fragment with a length of ~1200 base pairs encoding the full-length H4 receptor. DNA encoding a hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted after the start methionine codon using PCR mutagenesis. This DNA was ligated into the *Eco*RV site of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) and its sequence was determined.

Northern Expression Analyses. mRNA from various human and rat tissues were extracted as described previously (Marchese et al., 1994). Briefly, poly(A)$^+$ RNA was isolated using oligo(dT) cellulose spin columns (Pharmacia, Uppsala, Sweden), denatured and size fractionated on a 1% formaldehyde agarose gel, transferred onto nylon membrane, and immobilized by UV irradiation. The blots were hybridized with ^{32}P-labeled DNA fragments encoding the human and rat H4 receptor, washed with 2× standard saline/phosphate/EDTA and 0.1% SDS at 50°C for 20 min and with 0.1× standard saline/phosphate/EDTA and 0.1% SDS at 50°C for 2 h and exposed to X-ray film at −70°C in the presence of an intensifying screen.

Radioligand Binding Studies. For these studies, the HA-tagged H4 receptor was transiently transfected into human embryonic kidney 293 cells into 100-mm plates using FUGENE-6 (Roche

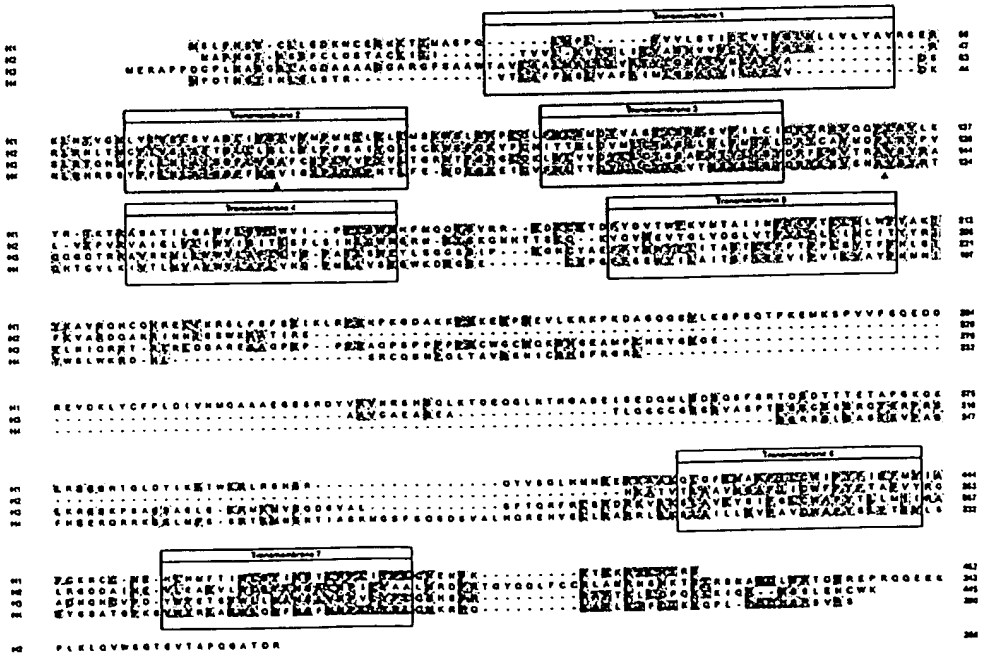

Fig. 1. Alignment of four human histamine receptors H1, H2, H3 and H4. Residues identical among the receptors are shaded. Numeric amino acid positions are indicated on the right. The presence of introns interrupting the H4 sequence are shown as "▲". H4 sequence data has been deposited in GenBank (accession number AY008280).

Molecular Biochemicals, Rotkreuz, Switzerland) at a 6:1 ratio of FUGENE/DNA as detailed previously (Shapiro et al., 2000). At 72 h after transfection, cells were harvested and membranes prepared as described previously (Shapiro et al., 2000); membranes were stored as pellets at −80°C until use. Radioligand binding assays were performed in a total volume of 500 μl in 50 mM Tris-Cl, 0.5 mM EDTA, pH 7.4, with 15 nM [^{3}H]pyrilamine in the 96-well format. After a 1-h incubation at room temperature, membranes were harvested by rapid filtration with a Brandel Harvester followed by two quick washes of ice-cold binding buffer. After drying, filters were placed into scintillation fluid and quantified by liquid scintillation spectrometry. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and data presented represents the mean ± S.E.M. of computer-derived estimates of at least three separate experiments, each done in duplicate. For inhibition studies, 12 concentrations of unlabeled ligand spanning 6 log units of test drug were used; for saturation studies, six concentrations of labeled ligand spanning 3 log units were used.

Immunoblot Analysis. The HA-tagged H4 receptor was transiently transfected into COS-7 monkey kidney cells (American Type Culture Collection, Manassas, VA) and membranes prepared from these cells as described previously (Lee et al., 2000). In brief, tissues were solubilized in sample buffer consisting of 50 mM Tris-HCl, pH 6.5, 1% SDS, 10% glycerol, 0.003% bromphenol blue, and 10% 2-mercaptoethanol. The samples were subjected to polyacrylamide gel electrophoresis with 12% acrylamide gels and electroblotted onto nitrocellulose as described previously (Ng et al., 1996). HA-tagged H4 immunoreactivity was revealed with the 3F10 rat monoclonal antibody (Roche, Laval, Quebec).

Internalization Studies. For these studies, HA-tagged H4 receptors were transfected into HEK-293 cells as described above into 100-mm plates. At 24 h after transfection, cells were split into 24-well plates containing poly-lysine-coated glass cover slips using Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum. The medium was replaced 24 h later with serum-free Dulbecco's modified Eagle's medium. The next day, cells were exposed to 100 μM histamine for 0, 2, 5, or 15 min and then fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then permeabilized on ice (0.2% Triton X-100 in ice-cold PBS) for 20 min and then incubated with blocking buffer (5% nonfat dry milk, 2% bovine serum albumin in PBS) for 1 h and then incubated with a 1:2000 dilution of monoclonal anti-HA antibody in blocking buffer overnight at 4°C. The next day, after extensive washing in room temperature PBS, cells were incubated with secondary antibody (BODIPY-labeled goat anti-mouse; 1:250 dilution in blocking buffer) for 1 h, washed extensively with PBS, and prepared for confocal microscopy as detailed previously (Berry et al., 1996; Kristiansen et al., 2000). Internalization was quantified as described previously (Berry et al., 1996; Willins et al., 1999).

Results and Discussion

As part of our ongoing search of novel genes encoding GPCRs, we queried the GenBank sequence databases maintained by NCBI with known GPCR sequences. A search with the histamine H3 receptor sequence retrieved partial sequences of a novel GPCR-encoding gene in an unfinished sequence of a human contig mapped to chromosome 18 (GenBank accession number AC007922). The retrieved sequence was obtained in three separate fragments, including one fragment that seemed to encode the receptor from the start methionine to TM2, another fragment encoding TM3, and a third fragment encoding TM5 through TM7 of a novel GPCR-like gene. These partial sequences were used to design primers for PCR amplification of human genomic DNA. Two frag-

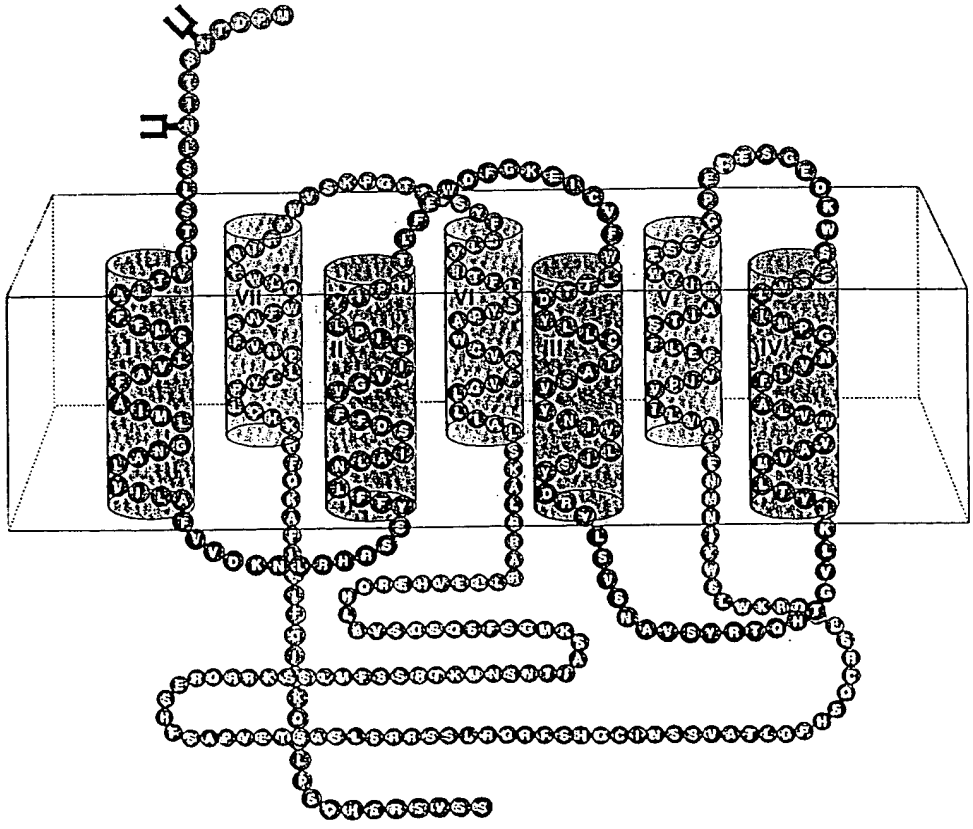

Fig. 2. Representative schematic of the human H4 receptor embedded in a cell membrane (box). Transmembrane regions are numbered and depicted with a top (extracellular)/bottom (intracellular) orientation. *N*-linked glycosylation sites are indicated in blue. Amino acids in red represent residues shared with the histamine receptor H3.

ments of DNA, encoding the start methionine to TM2 and TM5 to TM6, respectively, were obtained and used as probes to screen a human genomic library. The Met-TM2 probe retrieved two phages encoding the 5′ end of the novel gene from the start methionine to TM2, whereas the TM5-TM6 probe retrieved four phages encoding the 3′ end of the gene from TM5 to the stop codon.

Using the TM5-TM6 probe, we screened several tissues by Northern analyses, which revealed a 3.0-kb signal in rat testis. A human testis cDNA library was screened with both probes, which isolated two identical phages encoding the sequence from TM2 to TM6 of the gene. The sequences were identical in regions of overlap, confirming them to be partial sequences of the same gene. A comparison of the cDNA fragment to the GenBank genomic sequence revealed two introns interrupting the H4 receptor sequence. The first intron was ~8 kb in length and located within the TM2-encoding region (interrupting the translated sequence "LNLAISDFFVG. . . VISIPLYIPH"). The second intron was downstream in the region encoding the second intracellular loop (interrupting the translated sequence "DRYLSVSNA. . . VSYRTQHTGV"). The length of this intron could not be determined, because the GenBank genomic sequence was incomplete.

A BLASTX search of the GenBank database with this novel DNA sequence revealed the greatest overall identity with the histamine receptor H3 (~40%) and, more distantly, with other amine-type receptors of the GPCR family (<30%). As also reported with the H3 receptor (Lovenberg et al., 1999), this novel receptor shared greater identity with other amine receptors including the serotonin, adrenergic, dopamine, and muscarinic receptors (~ 25%) than the histamine receptors H1 or H2 (~20%). An alignment with the known histamine receptors (Fig. 1) revealed a higher sequence similarity to the H3 receptor (58% in the TM regions) than to the H1 and H2 receptors (26 and 27% in the TM regions, respectively). It has been determined from their sequence similarities and phylogenetic analyses that the members of the histamine receptor family may have evolved from different ancestral genes and, through convergent evolution, acquired the residues to recognize and bind histamine (Leurs et al., 2000). This novel receptor, which we have named H4, seems to be a novel member of the histamine receptor subfamily, with closest relation to the H3 receptor. The amino acid sequence of the H4 receptor revealed many conserved residues and motifs found within the GPCR family (Fig. 2). Among these is an aspartic acid residue in TM 3 that is conserved in all cationic amine receptors and has been shown to be important in binding various amines to GPCRs (Savarese and Fraser, 1992). In addition, the human Met-TM2 probe was used to screen a rat genomic library, which retrieved a phage encoding the 5′ end of the novel gene from the start methionine to TM2. This fragment shared 71% identity (83% in the TM regions) to the human H4 sequence, revealing a rat H4 ortholog.

The human Met-TM2 and TM5-TM6 probes were used in Northern analyses of various human and rat tissues. In the rat, the TM5-TM6 probe revealed a single transcript of 3 kb

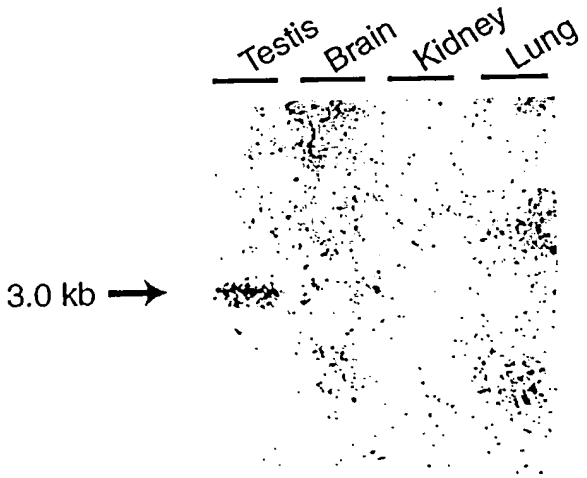

Fig. 3. Northern blot analysis of the distribution of H4 mRNA in various rat tissues. Each lane contained 10 μg of poly(A)⁺ RNA.

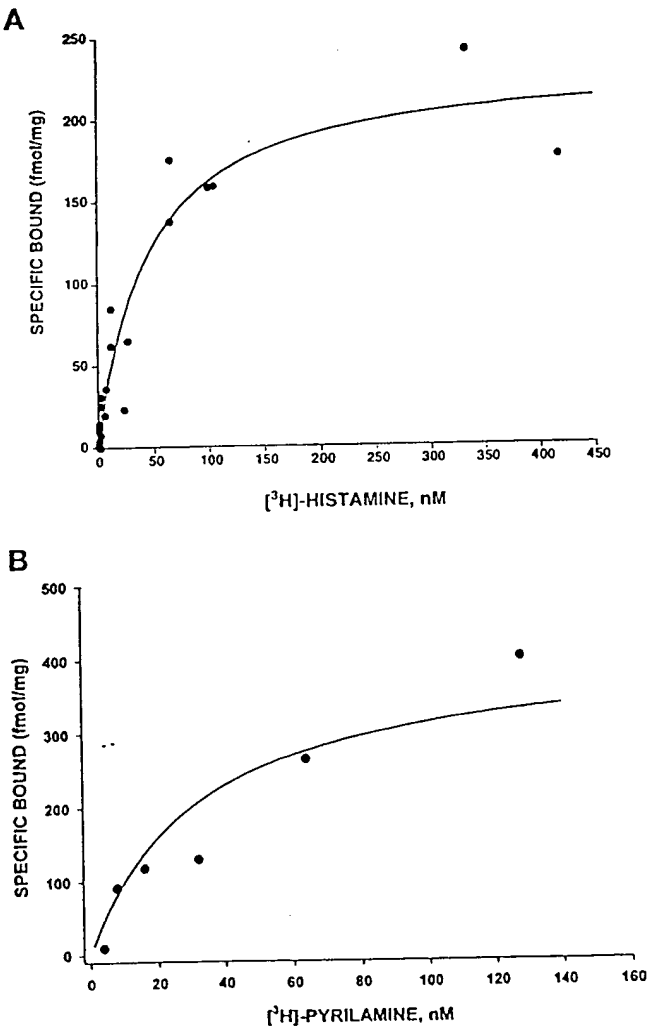

Fig. 4. Saturation binding studies of [³H]histamine (A) and [³H]pyrilamine (B) specific binding in membranes prepared from HEK-293 cells transiently transfected with H4 cDNA. Shown are typical results from experiment replicated three (A) and two times (B), with data representing mean of duplicate determinations. The curve represents the theoretical fit of the data A) (K_d = 44 nM; B_{max} = 235 fmol/mg) and B) (K_d = 32 nM; B_{max} = 437 fmol/mg). Nonspecific binding was determined with 100 μM mianserin.

in the testis (Fig. 3). The rat DNA fragment encoding from the start methionine to TM2 was used in Northern analyses of various rat tissues, revealing a 3-kb transcript in intestine (data not shown). The H3 and H4 receptors had significantly different mRNA expression distributions. H4 mRNA was detected in two peripheral tissues (with no detectable levels in brain or various peripheral tissues, including heart, stomach, small intestine, kidney, or liver). In contrast, H3 mRNA has been reported to be abundant in the brain (Lovenberg et al., 1999). Thus, H4 is not likely to be the H3-subtype characterized previously in brain tissue (West et al., 1990; Leurs et al., 1996), which suggests that yet another histamine receptor subtype remains unidentified.

To test the H4 receptor for pharmacological characterization, we constructed a full-length open reading frame by individually amplifying and joining the three H4-encoding fragments by PCR. The receptor was expressed in HEK-293 cells, and a variety of tritiated ligands including [^{3}H]histamine (histamine receptor nonselective), [^{3}H]pyrilamine (H1 receptor-selective), and [^{3}H]tiotidine (H2 receptor-selective) were tested against unlabeled ligands including mianserin, cyproheptadine, histamine, and clozapine. Specific binding obtained with [^{3}H]histamine (44 nM) or [^{3}H]pyrilamine (5–20 nM) and mianserin (100 μM) represented between 40 and 75% of total binding. Saturation binding studies performed with [^{3}H]histamine and [^{3}H]pyrilamine indicated respective K_d values of 44 and 32 nM and respective B_{max} values of 235 and 437 fmol/mg of membrane protein (Fig. 4). No specific [^{3}H]histamine or [^{3}H]pyrilamine binding was observed with untransfected HEK-293 cells.

Competition binding studies with various selective and nonselective histaminergic compounds were demonstrated. Typical data are shown in Fig. 5 and summarized in Table 1. The highest affinities were for amitriptyline and chlorpromazine, which are tricyclic compounds that have high affinity for the H1 histamine receptor (see on-line database at: *http://pdsp.cwru.edu/pdsp.asp*). Doxepin, cinnarizine, and promethazine (H1-selective antagonists) also displayed high affinity for the H4 receptor. Imetit (H3-selective agonist) and dimaprit (H2-selective agonist) had weak affinities for the H4 receptor, whereas mianserin (an H1 and H2 antagonist), cyproheptadine (a nonselective histamine/serotonin antagonist), and clozapine (an atypical antipsychotic drug with high affinity for a large number of receptors) had moderate affinities. The pharmacological profile of the H4 receptor is distinct from the histamine receptors (Table 1).

An HA epitope tag-encoding sequence was inserted after the start methionine for Western blot visualization. This plasmid was transiently transfected into COS-7 cells. Immunoblot analyses of membranes from these cells revealed high expression of the H4 receptor (Fig. 6A), with bands at 44 kDa, 85 kDa, and higher molecular mass species. The 44-kDa band matched the expected mass of the unglycosylated receptor and the 85-kDa band matched the glycosylated form. The bands >250 kDa represent oligomeric receptor species (Lee et al., 2000). The functional activity of the HA-tagged H4 receptor was examined by measuring histamine-induced internalization. As shown in Fig. 6B, exposure to 100 μM

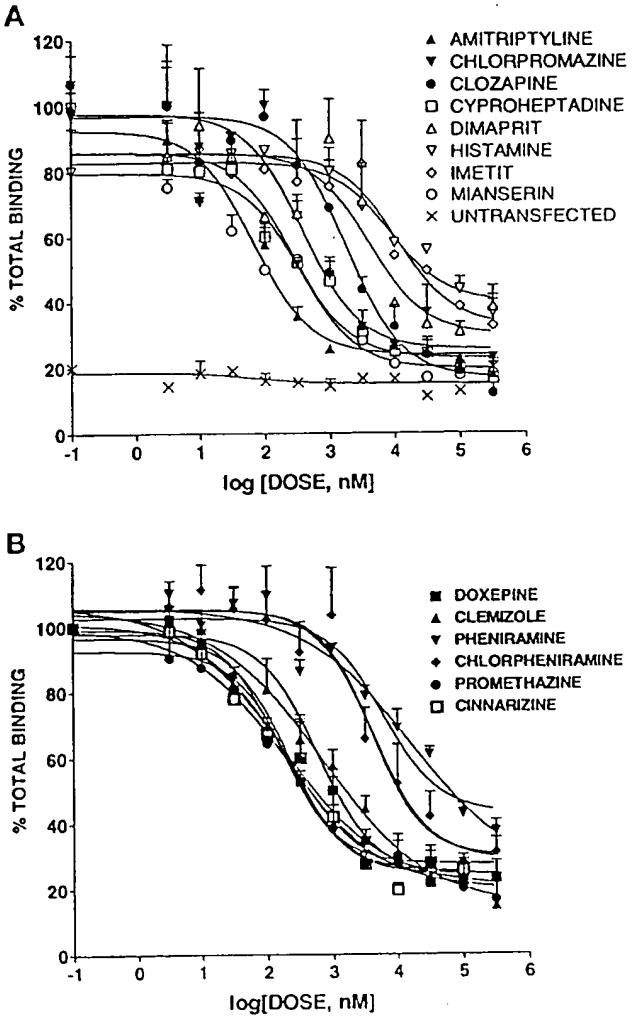

Fig. 5. Two sets (A and B) of competition binding of [^{3}H]pyrilamine with various ligands in membranes prepared from HEK-293 cells transiently transfected with H4 cDNA. Shown are typical results from competition binding experiments using 20 nM [^{3}H]pyrilamine and various concentrations of unlabeled ligands. Also shown are typical results obtained with untransfected HEK-293 cells studied in parallel (A).

TABLE 1

Ligand affinities for the H4 receptor

Data represent mean ± S.E. for two to four separate experiments. Twelve different concentrations spanning 6 log units of test drug were used to displace 15 nM [^{3}H]pyrilamine.

	$-\text{LogEC}_{50}$ (K_i)	Hill Coefficient
	nM	
Amitriptyline	7.31 ± 0.26 (33.6)	0.8
Chlorpromazine	7.13 ± 0.29 (50.2)	0.7
Doxepin	6.79 ± 0.13 (105.9)	0.6
Cinnarizine	6.73 ± 0.20 (141.6)	0.7
Promethazine	6.71 ± 0.19 (150.2)	0.6
Cyproheptadine	6.53 ± 0.09 (201.5)	0.9
Clemizole	6.16 ± 0.12 (402.2)	0.5
Mianserin	5.96 ± 0.40 (750.0)	0.9
Clozapine	5.90 ± 0.22 (849.6)	1.2
Chlorpheniramine	5.39 ± 0.16 (2910.0)	1.1
Histamine	5.30 ± 0.04 (3442.3)	0.5
Imetit	5.25 ± 0.44 (3795.6)	0.2
Pheniramine	5.21 ± 0.47 (4184.0)	0.2
Dimaprit	4.76 ± 0.19 (11812.7)	0.4
α-Methylhistamine	>10,000	Undetermined
Cimetidine	>10,000	Undetermined

histamine caused an internalization of H4 receptors from the plasma membrane to intracellular sites. Quantification revealed a rapid internalization of H4 receptors with significant internalization at 2 min after agonist exposure (Fig. 6C). These results indicated that the surface expression of the H4 receptor is functionally regulated by histamine exposure in a time-dependent fashion.

During the preparation of this article, other researchers (Oda et al., 2000) also reported the identification of a cDNA encoding a novel histamine receptor. This cDNA sequence varied from our sequence at three nucleotide positions, which translated into differences in three amino acids. Specifically, Ala138, His206, and Gln253 (as found in our sequence) were replaced by valine and two arginines, respectively. In the study by Oda et al., expression analyses revealed signals in peripheral blood leukocytes, small intestine, spleen and colon, and no expression in the brain. In addition, they reported histamine signaling through the novel receptor to be pertussis toxin-sensitive, suggesting a $G_{i/o}$ pathway of activation. For our receptor, we examined several second messenger-effector systems. We were not able to demonstrate H4 receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase, alterations in phosphoinositide hydrolysis, or mitogen-activated protein kinase phosphorylation (extracellular signal-regulated kinase 1/2 phosphorylation) in HEK-293 cells.

In conclusion, we report the discovery of a novel histamine receptor, H4. Previously, it was observed that histamine receptors shared greater sequence similarities with other biogenic amine-binding GPCRs than with one another. H4 shared highest sequence similarity with the previously reported histamine H3 receptor. In combination with the H1, H2, and H3 receptors, this receptor, with its unique distribution and pharmacology, will undoubtedly provide further insight into the physiological functions and therapeutic applications of this receptor family.

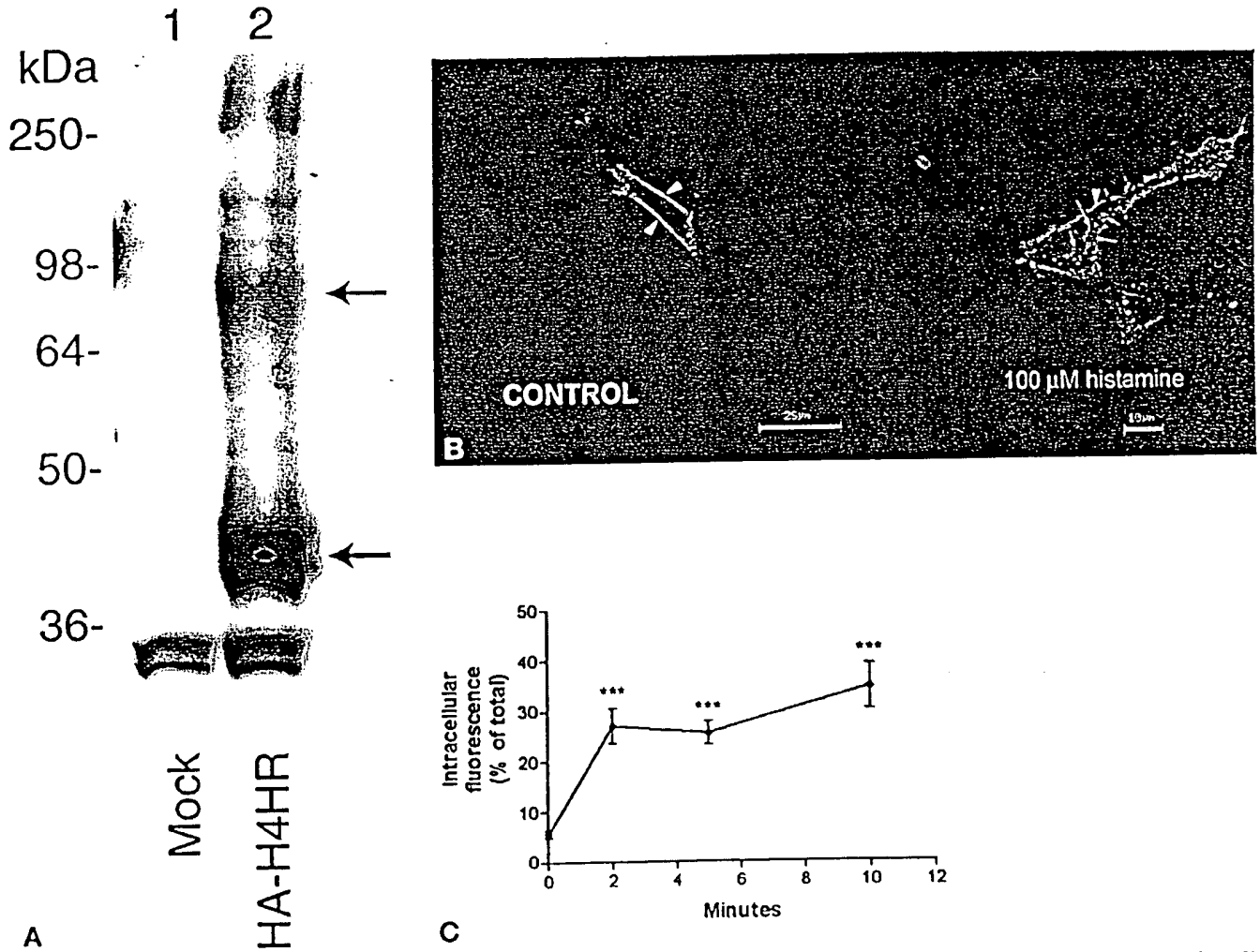

Fig. 6. A, immunoblot analysis of membranes from mock-transfected COS-7 cells (lane 1) and COS-7 cells expressing the HA-H4 receptor (lane 2). Membrane protein (25 μg) was used in each lane. Arrows indicate the unglycosylated (bottom) and glycosylated (top) receptor. B) histamine-induced internalization of the H4 receptor in HEK-293 cells. Shown are representative confocal micrographs in which HA-tagged H4 receptors were examined in cells exposed to vehicle (PBS) or 100 μM histamine for 5 min and then prepared for microscopy as described previously (Berry et al., 1996; Willins et al., 1999; Kristiansen et al., 2000). Arrowheads and arrows indicate cell-surface and internalized receptors, respectively. C, histamine-induced time-dependent internalization of the H4 receptor in HEK-293 cells. Shown are the mean ± S.E.M. of the percentage internalization of HA-tagged H4 receptors in HEK-293 cells (n = 20–30 cells/time point) after exposure to histamine (100 μM) for various time periods. ***significantly different compared with 0-min value ($P < 0.0001$).

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In re: Glucksmann *et al.*
Appl. No. 09/464,685
Filed: December 16, 1999

APPENDIX C

CANCER GENETICS

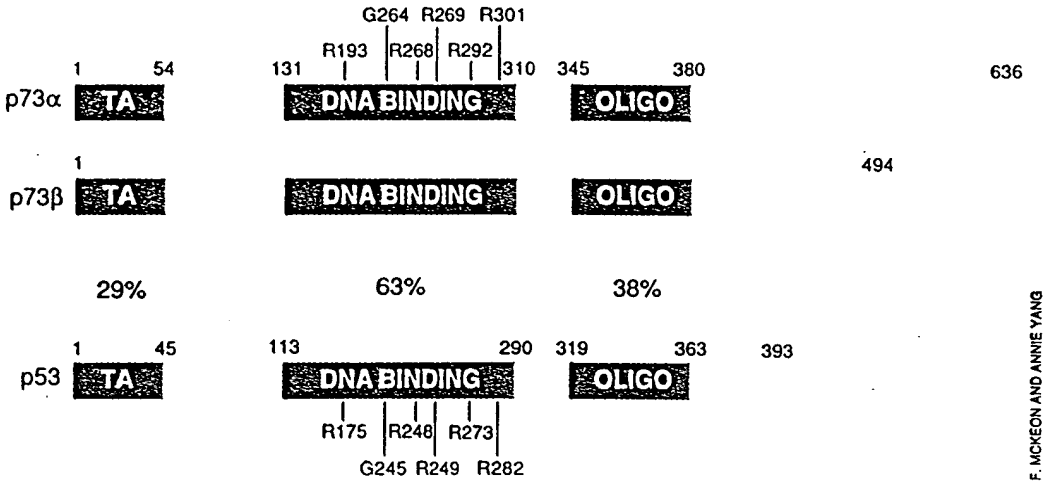
First *p53* Relative May Be a New Tumor Suppressor

In the 20 years since its discovery, the *p53* gene has become one of the most heavily scrutinized genes in history. Indeed, it's referenced in over 8000 papers in Medline, the online biomedical abstract service. The fascination is easy to understand: Loss or inactivation of *p53*, which is a so-called tumor-suppressor gene, is thought to contribute to the development of 50% of all human cancers. All that time, *p53* was thought to be an only child, with no close relatives. Now, researchers have discovered a new gene, a long-lost cousin called *p73*, that bears a strong resemblance to *p53*.

It is being greeted with the same surprise as any newfound relative. "Given the intense interest in this area, the fact that [*p73*] slipped through the cracks is surprising," says cancer biologist Tyler Jacks of the Massachusetts Institute of Technology (MIT). But the new gene should generate some intense interest of its own, because its protein not only resembles the p53 protein, but also seems to have similar activities. The p53 protein acts as a "security guard," deployed when a cell's DNA is damaged to prevent the cell from becoming cancerous. It does this by either inhibiting cell growth until the damage is repaired or causing the cell to commit suicide through a process called programmed cell death or apoptosis. The p73 protein appears to share these growth-inhibiting and apoptosis-promoting effects, although what triggers them and exactly what its cellular role is are both unknown.

Those findings, together with *p73*'s location in a region of chromosome 1 that is often deleted in cancers including neuroblastoma, a malignant tumor of nervous tissue, suggest that it, too, may be a tumor suppressor. "This [discovery] will titillate a whole lot of people," predicts cancer geneticist Bert Vogelstein of Johns Hopkins University School of Medicine, a pioneer of *p53* research. Indeed, if *p73* can stand in for *p53* when that gene is lost, it might be possible to design new cancer drugs that work by turning on *p73* in tumors lacking *p53*.

Molecular biologist Daniel Caput and his colleagues at the pharmaceutical company Sanofi Recherche in Labege, France, identified the *p73* gene while looking for something completely different, namely genes that respond to certain immune system regulators. When the French team sequenced the many potential targets their screen had turned up, they were shocked to find that one false positive had remarkable similarities to *p53*.



F. MCKEON AND ANNIE YANG

No longer alone. The p73 proteins, although longer, resemble p53 in three regions: the transcription activation (TA, 29% identical) and DNA binding domains (63% identical) and also the domain where p53 binds itself (OLIGO, 38% identical). The labeled amino acids indicate residues that are frequently mutated in *p53* and are conserved in *p73*.

As the researchers report in the 22 August issue of *Cell*, the proteins made by *p73* are somewhat larger than p53. But they found that one section of p73 closely resembles the so-called "core binding region" of p53. Many of p53's activities depend on its ability to regulate other genes, and the core binding region is where the protein attaches itself to the DNA when exerting its effects. Of 177 amino acids in that region, 112 are identical. Additional similarities turned up in two other sections thought to be involved in p53 activity—one needed for its gene regulatory effects and another where it apparently binds to itself. These resemblances are enough to suggest that the two genes are the progeny of a gene that was duplicated in some ancient cellular event. Indeed, *p73* may be the ancestral gene, because a gene found in squid that was supposed to be that species's version of *p53* is actually more similar to *p73*.

"This [discovery] will titillate a whole lot of people."

—Bert Vogelstein

The structural similarities between p53 and p73 also suggested that the proteins might have similar roles in the cell. So Caput and his colleagues joined forces with their longtime collaborator, cell biologist Frank McKeon, who studies gene expression and cell division at Harvard Medical School in Boston, to look for parallels. One way p53 restrains cells that have damaged DNA is by triggering the production of a protein called p21, which blocks cell division. The Caput-McKeon team found that adding *p73* to a line of neuroblastoma that lacks the gene also triggers p21 production, an indication that p73 inhibits cell growth through the same pathway used by p53.

In a paper that appears in this week's issue of *Nature*, molecular biologist William Kaelin at the Dana-Farber Cancer Institute in Boston and his colleagues report similar findings with another tumor cell line. Kaelin's team also found evidence that p73 can mimic p53's ability to cause cell suicide. When overexpressed in these cells, p73 latched onto stretches of DNA to which p53 normally attaches itself when instructing a cell to self-destruct.

Together, the findings suggest that *p73*, too, may be a tumor suppressor, an idea that is buttressed by its provocative chromosomal location. The Caput-McKeon team found *p73* in a region near the tip of chromosome 1 that was already suspected of harboring one or more tumor suppressor genes, because the region is often missing in tumor cells.

The teams did find one major point of difference between the two genes, however. Unlike p53 protein, p73 does not seem to be produced in response to DNA damage. That implies that the protein is not a cell "security guard" the way p53 is. Early results of experi-

ments in which McKeon and Caput deleted the *p73* gene in mice suggest another possibility: It may be "developmentally important," he says, especially in the brain and immune system, although how remains to be clarified.

If *p73* is a tumor suppressor, it may behave somewhat differently than *p53* and other previously discovered tumor suppressors. Classic tumor-suppressor genes require two "hits" to be inactivated—a partial or complete deletion of one of the two gene copies, for example, and another, lesser change that cripples the second copy. But Caput, McKeon, and their colleagues have evidence that one *p73* copy is already inactive in normal cells—the apparent result of a mysterious process called imprinting. Its precise function isn't known, but during embryonic development, imprinting alters certain genes so that the copy inherited either from the mother or the father is specifically shut down.

If one *p73* copy has been silenced by imprinting, then only one hit—loss of the active copy—might be all that it takes to tip a cell into the uncontrolled growth of cancer. Says Kaelin, "*p73* may be the first example of a new paradigm for how tumor-suppressor genes are involved in cancer."

Indeed, molecular biologist Rogier Versteeg of the Academic Medical Center in Amsterdam, the Netherlands, has evidence that an imprinted gene may be involved in neuroblastoma development. He has identified two sites of chromosome damage that contribute to neuroblastoma by knocking out as-yet-undiscovered tumor-suppressor genes. Both lie in the same region of chromosome 1 where *p73* is located, and one illustrates "a strong bias" toward loss from the maternal copy of the chromosome in the cancer cells. This bias implies that this specific copy is the active one and must be lost to cause the cancer.

Other work from the Caput-McKeon team suggests that this mystery tumor-suppressor gene may be *p73*. When they looked for the gene in neuroblastoma cell lines, they found that one *p73* copy had been lost. And while they couldn't uncover any mutations in the remaining copy, most of the cell lines made no detectable p73 protein, implying that the second copy had been silenced by imprinting.

In spite of the differences in the roles of *p53* and its new cousin, both in normal cells and in cancer, the family resemblances may be strong enough for them to substitute for each other. If so, says MIT's Jacks, cancer might be treated by finding a way to switch on *p73* in tumor cells that have lost *p53*. "Even if *p73* is not normally involved in tumor suppression, maybe it could be recruited," says Jacks. Now McKeon and Caput are searching for further family members. But the discovery of *p73* is already certain to captivate their peers.

–Steven Dickman

Steven Dickman is a free-lance writer in Cambridge, Massachusetts.

AIDS RESEARCH

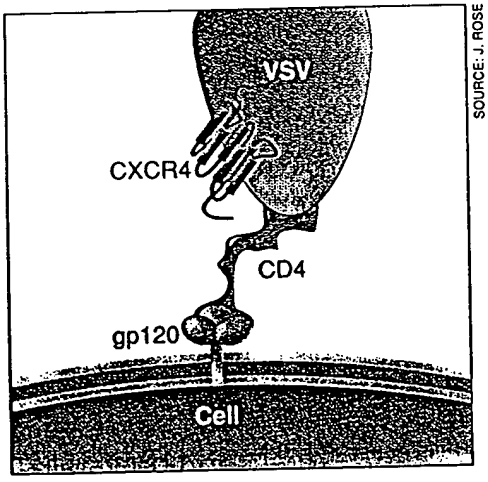
HIV Gets a Taste of Its Own Medicine

In an attempt to fight fire with fire, researchers have engineered a virus that usually infects cattle to attack the AIDS virus in humans. The innovative approach has so far shown promise only in test-tube experiments, but it is attracting widespread attention among AIDS researchers. "It's really on the verge of a breakthrough," says Nava Sarver, who oversees development of novel AIDS treatments at the National Institute of Allergy and Infectious Diseases (NIAID).

Yale University virologist John Rose and co-workers describe in the 5 September issue of *Cell* how they have constructed a potential HIV treatment by modifying vesicular stomatitis virus (VSV), which farmers detest because it causes a mouth infection in cattle that prevents them from eating. As the Yale researchers' experiments show, their newfangled VSV selectively targets and destroys HIV-infected human cells. "It's a pretty interesting way of harnessing a virus for peaceful purposes," says the University of Pennsylvania's Robert Doms. "It's a very clever approach."

The work builds on recent discoveries made by Doms and others about a two-part handshake between HIV and the cells it infects. After HIV binds to the CD4 receptor on a white blood cell, it also must link to another molecule found on the cell's surface, known as a chemokine receptor. Once these handshakes are complete, HIV gains entry, and shortly thereafter, new virus proteins make their way to the cell's outer coating, where they stick out like a flag of victory.

Rose and colleagues reasoned that if VSV could be induced to express these receptors on its surface, they would bind to the HIV proteins displayed on infected cells, turning VSV into a kind of guided missile. To test this idea, the researchers stitched into VSV the genes that code for CD4 and one of HIV's favored chemokine receptors, CXCR4, and added their engineered VSV to a culture containing HIV-infected cells. The virus did, indeed, target just the infected cells, killing them rapidly. "VSV is so fast," says Rose—much faster than HIV, he notes.



SOURCE: J. ROSE

Trojan horse. CD4 and CXCR4 receptors expressed by genetically engineered VSV bind to HIV's gp120 protein on surface of infected cell.

A potential downside to this approach is that the modified VSV might kill cells that aren't infected by HIV. Rose believes that won't happen because he has stripped VSV of its own surface protein, which is what allows it to infect a broad range of cells. "Without its normal coat, it can't infect anything," says Rose. But only animal tests will provide evidence of that, cautions NIAID director Anthony Fauci.

Although Fauci has high praise for the concept's ingenuity, he is concerned that it might take an impracticably high dose of the modified VSV to make a real dent in a person's HIV levels. Another worry, says monkey researcher Ronald Desrosiers of the New England Regional Primate Research Center in Southborough, Massachusetts, is that the body will quickly develop an immune response against VSV, limiting its ability to attack HIV.

Still, Sarver, Fauci, and others are anxious for Rose and colleagues to put their viral guided missile to more stringent test-tube and animal tests. Desrosiers already has begun working with Rose to test the concept in monkeys that have been infected with SIV, HIV's simian cousin. Desrosiers expects to have results in the next few months. Even if they are positive, however, human trials will require the approval of the Food and Drug Administration, which has shown great caution in the past about putting potentially therapeutic viruses into people.

Rose's strategy is not limited to attacking HIV. NIAID's Sarver suggests that if researchers can swap different receptors into this "gutted" VSV, the precisely targeted viruses could be used in everything from vaccines to gene therapies to cancer treatments. "We're not there yet," says Sarver, "but the potential applications are enormous."

–Jon Cohen

In re: Glucksmann *et al.*
Appl. No. 09/464,685
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APPENDIX D

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An Orphan Nuclear Receptor Activated by Pregnanes Defines a Novel Steroid Signaling Pathway

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Summary

Steroid hormones exert profound effects on differentiation, development, and homeostasis in higher eukaryotes through interactions with nuclear receptors. We describe a novel orphan nuclear receptor, termed the pregnane X receptor (PXR), that is activated by naturally occurring steroids such as pregnenolone and progesterone, and synthetic glucocorticoids and antiglucocorticoids. PXR exists as two isoforms, PXR.1 and PXR.2, that are differentially activated by steroids. Notably, PXR.1 is efficaciously activated by pregnenolone 16α-carbonitrile, a glucocorticoid receptor antagonist that induces the expression of the CYP3A family of steroid hydroxylases and modulates sterol and bile acid biosynthesis in vivo. Our results provide evidence for the existence of a novel steroid hormone signaling pathway with potential implications in the regulation of steroid hormone and sterol homeostasis.

Introduction

Steroid hormones are cholesterol derivatives that serve as signaling molecules to coordinate the expression of complex gene programs in higher eukaryotes. These molecules exert their effects by diffusing into cells and interacting with specific intracellular receptors. Receptors for each of the major classes of sex and adrenal steroids have been characterized (Evans, 1988; Beato et al., 1995; Mangelsdorf et al., 1995). In the absence of their cognate ligands, the steroid hormone receptors remain sequestered in the cytoplasm through interactions with large multiprotein complexes containing heat shock proteins. However, the binding of ligand causes the steroid hormone receptors to be released from these complexes and translocated into the nucleus (Pratt, 1993). Once inside the nucleus, the activated receptors regulate the expression of target genes by binding as homodimers to short DNA sequence motifs, termed hormone response elements (HREs) (Glass, 1994). In this manner, the steroid hormone receptors function as ligand-activated transcription factors.

The molecular cloning of steroid hormone receptors revealed that they comprise a subfamily within a larger superfamily of structurally related proteins (Evans, 1988; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). This superfamily also includes receptors for nonsteroidal, lipophilic molecules such as thyroid hormone, retinoids, fatty acids, and eicosanoids. The nonsteroid receptors differ from their steroid hormone receptor counterparts in several respects (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). First, the nonsteroid hormone receptors are not sequestered in the cytoplasm in the absence of their cognate ligands but instead reside within the nucleus. Second, whereas steroid hormone receptors generally bind to their HREs as homodimers, most of the nonsteroid hormone receptors identified to date bind to DNA as heterodimers with the 9-*cis* retinoic acid receptors (RXRs) (Glass, 1994; Mangelsdorf and Evans, 1995). Finally, the two classes of receptors recognize different types of HREs: steroid hormone receptors generally bind to HREs composed of two half-sites organized as a palindrome with a three-nucleotide spacer, while nonsteroid hormone receptors preferentially bind to HREs composed of two half-sites organized as a direct repeat (DR), with the number and composition of the nucleotides separating the half-sites serving as important determinants of receptor selectivity (Umesono et al., 1991; Glass, 1994; Mangelsdorf and Evans, 1995).

In addition to receptors with established ligands, approximately 30 other members of the nuclear hormone receptor family have been isolated from vertebrates. These related proteins lack identified ligands and, as a consequence, have been termed orphan nuclear receptors (Evans, 1988; Mangelsdorf and Evans, 1995; Enmark and Gustafsson, 1996). The search for hormonal activators of the orphan receptors has created exciting opportunities to discover novel endocrine signaling pathways with implications in both normal physiology and disease. Work with orphan nuclear receptors has led to the identification of fatty acids and eicosanoids as ligands for the peroxisome proliferator-activated receptors (Forman et al., 1997; Kliewer et al., 1997; Krey et al., 1997), retinoids and farnesoids as activators of RIP14/FXR (Forman et al., 1995; Zavacki et al., 1997), and various oxysterols as activators of the LXR and SF-1 orphan nuclear receptors (Janowski et al., 1996; Lala et al., 1997; Lehmann et al., 1997).

In this report, we describe a novel orphan nuclear receptor, which we have designated PXR for pregnane X receptor. Like nonsteroid hormone receptors, PXR binds as a heterodimer with RXR to an HRE composed of two half-sites organized as a DR. Surprisingly, however,

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PXR is efficaciously activated by several steroids, including naturally occurring pregnanes and synthetic glucocorticoids and antiglucocorticoids. Thus, PXR combines features of both the steroid and nonsteroid nuclear receptors. We suggest that PXR defines a novel steroid hormone signaling pathway that may account for at least some of the effects of synthetic glucocorticoids and antiglucocorticoids that do not appear to be mediated through the classical glucocorticoid receptor (GR) signaling pathway.

Results

Cloning of PXR.1 and PXR.2

In an effort to identify new members of the nuclear receptor family, we performed a series of motif searches of public EST databases. These searches revealed a clone from a mouse liver library in the Washington University/HHMI EST database that had homology to the ligand-binding domains (LBDs) of a number of nuclear receptors. We used this partial sequence information to isolate larger clones from a mouse liver cDNA library. The nucleotide sequence of the longest cDNA clone encodes a novel orphan nuclear receptor of 431 amino acids that we have designated PXR.1 (Figure 1A). We also isolated a second cDNA clone, termed *PXR.2*, that was identical to *PXR.1* except for the deletion of a stretch of 123 nucleotides extending from base pairs 661 to 783 (Figure 1A). Examination of the *PXR* genomic structure revealed that *PXR.2* represents a splice variant of *PXR.1* lacking a single exon (data not shown). The *PXR.2* cDNA encodes a 390 amino acid protein that lacks a 41 amino acid region in the putative LBD of PXR.1. Sequence alignment with nuclear receptors for which the crystal structures have been solved indicates that the 41 amino acids that distinguish the PXR isoforms lie between helices two and three of the canonical LBD structure (Wurtz et al., 1996).

Sequence comparison with other members of the nuclear receptor family showed that the PXR isoforms are most closely related to the *Xenopus laevis* orphan nuclear receptor 1 (ONR1) (Smith et al., 1994), with PXR.2 and ONR1 sharing 70% and 46% amino acid identity in their DNA-binding domains (DBDs) and LBDs, respectively (Figure 1B). Based upon this degree of homology, it is unclear at present whether PXR represents the mammalian homolog of ONR1. With regard to mammalian receptors, PXR is most closely related to the vitamin D receptor (VDR) (Baker et al., 1988), sharing 64% and 39% identity in the DBD and LBD, respectively.

PXR Expression Pattern in the Embryo and the Adult

The expression pattern of PXR was examined in both adult and embryonic tissues. Northern blot analysis was performed under high-stringency conditions with blots that included poly(A)$^+$ RNA prepared from multiple adult mouse tissues and a probe that recognized both PXR isoforms. Abundant expression of *PXR* mRNA was observed only in the liver and intestine, where three distinct messages were detected including a highly expressed mRNA species of 2.6 kb and two transcripts of 1.9 kb and 4.4 kb that were expressed at lower levels (Figure 2A). Weaker expression of the *PXR* mRNA was also detected in kidney and stomach (Figure 2A). No *PXR* mRNA was detected in the other tissues examined.

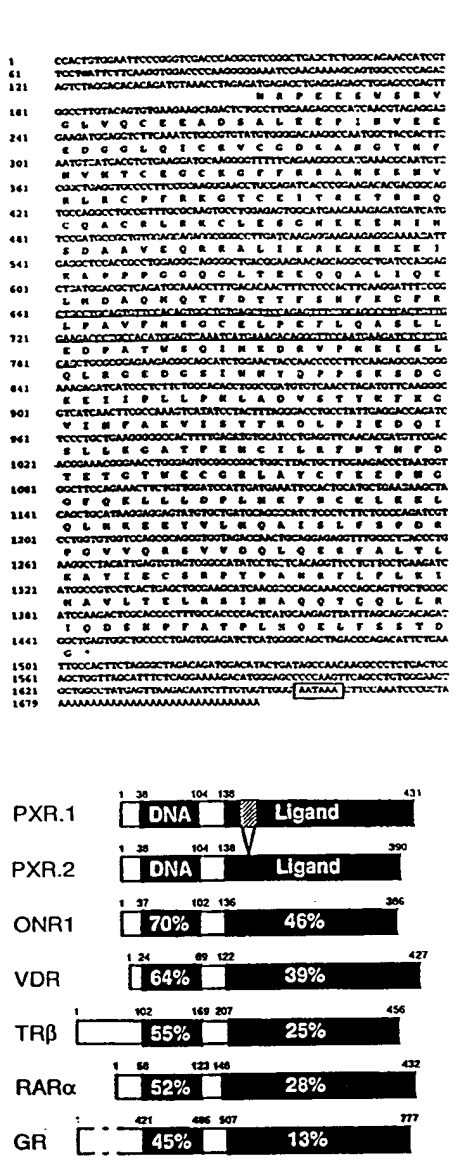

Figure 1. PXR is a Member of the Nuclear Receptor Superfamily

(A) Nucleotide and predicted amino acid sequence of mouse PXR. The upstream in-frame stop codon is in bold. The predicted start initiation codon is at nucleotide 151. The 123 nucleotide region extending from base pairs 661 to 783 that is present in PXR.1 and absent in PXR.2 is underlined. A putative polyadenylation signal in the 3′ untranslated region is boxed.

(B) Amino acid sequence comparison between murine PXR and other members of the nuclear hormone receptor family. Similarity between PXR and other nuclear hormone receptor family members in the DNA- and ligand-binding domains are indicated as percent amino acid identity. The 41 amino acid region that distinguishes PXR.1 from PXR.2 is indicated by the cross-hatched area. All comparisons were made with the PXR.2 isoform. ONR1, *Xenopus* orphan nuclear receptor 1; VDR, human vitamin D receptor; TRβ, human thyroid hormone receptor β; RARα, human retinoic acid receptor α; GR, human glucocorticoid receptor.

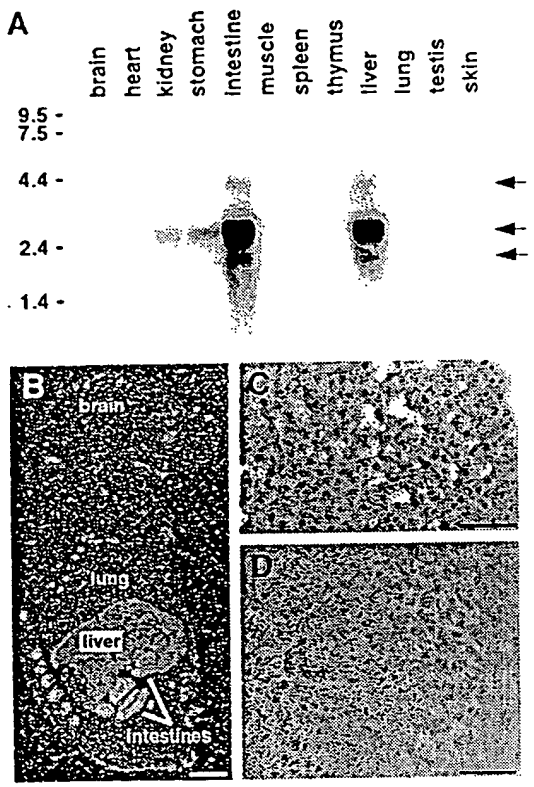

Figure 2. Expression Pattern of PXR in Adult and Embryonic Tissues

(A) Northern blot analysis. RNA size markers (in kb) are indicated at left. Arrows at right indicate the three species of *PXR* transcripts that were detected. (B-D) In situ hybridization analysis of *PXR* mRNA expression in E18 mouse embryo sections with a probe that recognized both PXR isoforms (see probe 1 in Experimental Procedures). (B) Phosphoimage of an entire sagittal section showing specific labeling in intestine and liver. Specificity was ascertained by competition with a 100-fold excess of unlabeled, specific oligonucleotide. This resulted in complete inhibition of labeling in liver and intestine while nonspecific signals remained in developing bone. (C and D) High power bright-field microscopy showing the abundance of silver grains in the liver (C) and in the intestine (D) where mRNA labeling was confined to the epithelium. The bar in (B) corresponds to 2 mM, and in (C) and (D) to 50 μM.

The embryonic expression pattern of *PXR* was examined via in situ hybridization analysis using sections prepared from day 18 (E18) mouse embryos. Three oligonucleotide probes were designed that recognized *PXR*, including two that interacted with both *PXR* isoform mRNAs and a third that hybridized only to *PXR.1* mRNA. *PXR* mRNA was detected in the liver and intestine (Figures 2B–2D). Staining in the intestine was confined to the epithelium (Figure 2D). No *PXR* expression was detected in embryonic kidney, adrenal, lung, thymus, heart, skeletal muscle, brain, or spinal cord (data not shown). While similar results were obtained with all three *PXR* probes, the signal intensity in liver and intestine was consistently higher in experiments performed with the probes that recognize both *PXR* isoforms (data not shown), suggesting that *PXR.2* is expressed in tissues that also express *PXR.1*. We conclude from the Northern blot and in situ hybridization analyses that *PXR* is abundantly expressed in only a few tissues, including the liver and intestine, in both the mouse embryo and adult.

PXR Is Activated by Synthetic Pregnanes and Glucocorticoids

We next sought to determine whether PXR, like other members of the nuclear receptor family, possesses transcriptional activity that can be regulated in a hormone-dependent manner. As we lacked knowledge of a cognate HRE for PXR, we initially performed searches for activators using an established chimera system in which the LBDs of the two PXR isoforms were fused to the DBD of the yeast transcription factor GAL4 (Lehmann et al., 1995). Expression vectors for the GAL4-PXR chimeras were transiently transfected into CV-1 cells together with a reporter plasmid containing five copies of a GAL4 DNA binding site upstream of the chloramphenicol acetyltransferase (CAT) reporter. Transfected CV-1 cells were systematically treated with a series of natural and synthetic compounds that included steroids, vitamin D analogs, thyroid hormone analogs, retinoids, fatty acids, and other small, lipophilic molecules, and reporter levels measured.

Interestingly, we found that the activity of the GAL4-PXR.1 chimera was markedly induced by 10 μM concentrations of a variety of synthetic steroids including the glucocorticoids dexamethasone, dexamethasone-t-butyl-acetate, and dexamethasone-21-acetate, and the pregnenolone derivative 6,16α-dimethyl pregnenolone (Figure 3B). Dexamethasone-t-butylacetate and 6,16α-dimethyl pregnenolone were the most efficacious of these compounds (Figure 3B). Remarkably, we found that PXR.1 was not only activated by GR agonists, but also by the GR antagonists RU486 and pregnenolone 16α-carbonitrile (PCN) (Figure 3B). RU486 is a potent antiprogestin that binds to the GR at nanomolar concentrations (Cadepond et al., 1997). PCN is a weaker antagonist that has been shown to interfere with GR-mediated activation at micromolar concentrations (Schuetz and Guzelian, 1984; Schuetz et al., 1984). Thus, the LBD of PXR.1 was efficaciously activated by both agonists and antagonists of the GR.

Notably, the GAL4-PXR.2 chimera displayed a much more restricted activation profile. Of the steroids that activated GAL4-PXR.1, only dexamethasone-t-butyl-acetate activated GAL4-PXR.2 efficiently (Figure 3B). We conclude that the 41 amino acid deletion that distinguishes PXR.2 from PXR.1 has a marked effect on the responsiveness of the orphan receptor to synthetic steroids.

PXR Functions through a Response Element Conserved in the *CYP3A* Gene Promoters

PCN and dexamethasone treatment have previously been shown to induce the expression of the *CYP3A* family of genes in rodent liver, intestine, and kidney as well as in primary cultures of rodent hepatocytes (Elshourbagy and Guzelian, 1980; Heuman et al., 1982; Hardwick et al., 1983; Schuetz and Guzelian, 1984; Schuetz et al., 1984; Gonzalez et al., 1985; Debri et al., 1995). The *CYP3A* genes encode cytochrome P450 hemoproteins involved in the hydroxylation of steroid hormones, including corticosteroids, progestins, androgens, and DHEA-sulfate, as well as a variety of xenobiotics (Nebert and Gonzalez, 1987; Juchau, 1990). The response to PCN and dexamethasone occurs at the level

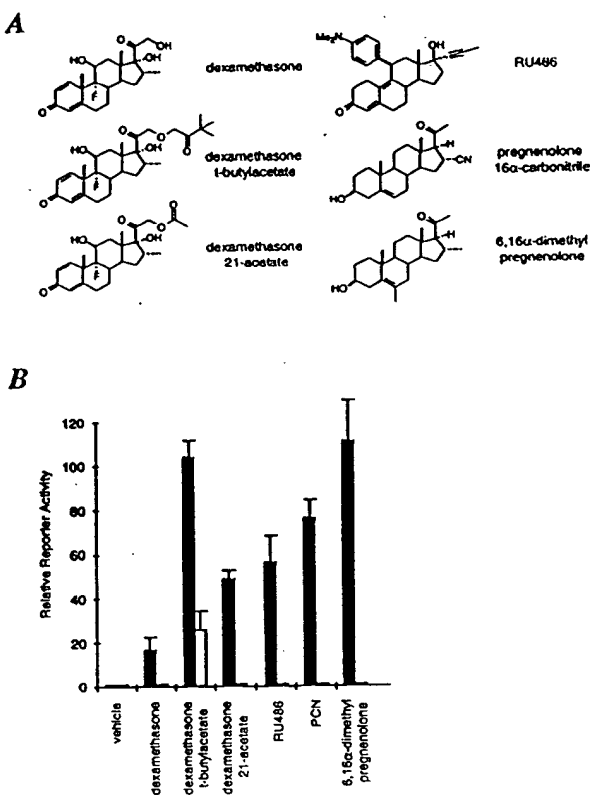

Figure 3. Synthetic Glucocorticoids and Pregnenolone Derivatives Activate PXR

(A) Structures of the steroids that activate the GAL4-PXR chimeric proteins.

(B) CV-1 cells were cotransfected with expression plasmids encoding either GAL4-PXR.1 (filled bars) or GAL4-PXR.2 (open bars) and the reporter plasmid *(UAS)$_5$-tk-CAT*. Cells were treated with vehicle alone (0.1% DMSO) or 10 μM of the indicated steroids. Cell extracts were subsequently assayed for CAT activity. Data represent the mean of five data points from two different experiments ± SD.

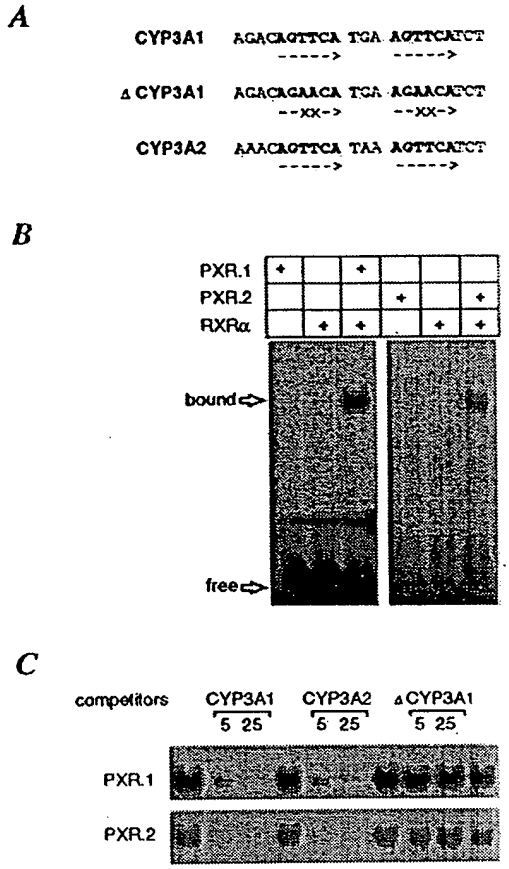

Figure 4. PXR Binds as a Heterodimer with RXRα to DR-3 Response Elements Present in the *CYP3A1* and *CYP3A2* Gene Promoters

(A) Alignment of DR-3 motifs present in the promoter regions of the *CYP3A1* and *CYP3A2* genes. A mutated derivative of the *CYP3A1* DR-3 motif (*ΔCYP3A1*) used in the gel mobility shift assays and the position of the mutations is also shown.

(B) Gel mobility shift assays were performed with a radiolabeled oligonucleotide containing the *CYP3A1* DR-3 and in vitro synthesized PXR.1, PXR.2, and RXRα as indicated.

(C) Gel mobility shift assays were performed with PXR.1 and RXRα (top panel) or PXR.2 and RXRα (bottom panel) and radiolabeled oligonucleotide containing the *CYP3A1* DR-3 in the presence of either a 5-fold or 25-fold excess of unlabeled oligonucleotides containing the *CYP3A1* DR-3, a mutated *CYP3A1* DR-3 (*ΔCYP3A1*), or the *CYP3A2* DR-3 as indicated.

of transcription and has been mapped to a conserved region within the *CYP3A1* and *CYP3A2* gene promoters that does not contain a typical glucocorticoid response element, but instead contains a DR of the nonsteroid nuclear receptor half-site sequence AGTTCA separated by a three-nucleotide spacer, a so-called DR-3 motif (Figure 4A) (Umesono et al., 1991; Miyata et al., 1995; Quattrochi et al., 1995; Huss et al., 1996). These data have led to speculation that a nonsteroid nuclear hormone receptor might be involved in mediating the effects of PCN and dexamethasone.

Given that PXR shares a high degree of homology with ONR1 and VDR in the DBD (Figure 1B) and that both ONR1 and VDR preferentially bind to DR-3 HREs as heterodimers with RXR (Umesono et al., 1991; Smith et al., 1994; Mangelsdorf and Evans, 1995), we postulated that PXR might bind to the *CYP3A1* and *CYP3A2* DR-3 motifs as a heterodimer with RXR. In order to test this idea, gel mobility shift assays were performed using a radiolabeled oligonucleotide containing the *CYP3A1* DR-3 motif and in vitro synthesized PXR.1, PXR.2, and RXRα. Neither PXR nor RXRα bound to the *CYP3A1* DR-3 alone. However, both PXR.1 and PXR.2 bound efficiently to the *CYP3A1* DR-3 as heterodimers with RXRα (Figure 4B). The PXR-RXRα complex with DNA was competed efficiently by an excess of unlabeled *CYP3A1* DR-3 oligonucleotide or an oligonucleotide containing a closely related DR-3 motif from the *CYP3A2* gene promoter (Figures 4A and 4C). No competition was seen with an oligonucleotide containing a mutated *CYP3A1* DR-3 motif (Figures 4A and 4C). Thus, both PXR isoforms can bind specifically as heterodimers with RXRα to DR-3 motifs found in the promoter regions of *CYP3A* genes.

We next asked whether the PXR isoforms could induce gene expression through the *CYP3A1* DR-3 element in response to steroids. Transient transfection assays were performed with a reporter plasmid containing two copies of the *CYP3A1* DR-3 motif inserted

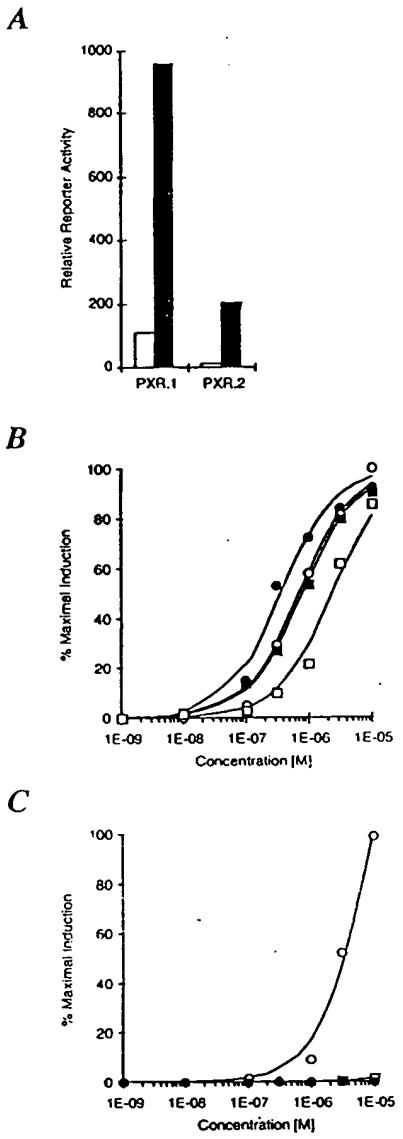

Figure 5. Synthetic Glucocorticoids and Pregnenolone Derivatives Activate PXR through the *CYP3A1* DR-3

(A) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 or PXR.2 and the *(CYP3A1)$_2$-tk-CAT* reporter plasmid. Cells were treated with either vehicle (0.1% DMSO) alone (open bars) or 10 μM dexamethasone-t-butylacetate (filled bars). Cell extracts were subsequently assayed for CAT activity.

(B and C) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 (B) or PXR.2 (C) and the *(CYP3A1)$_2$-tk-CAT* reporter plasmid. Transfected cells were treated with the indicated concentrations of 6,16α-dimethyl pregnenolone (closed circles), dexamethasone-t-butylacetate (open circles), PCN (closed squares), or RU486 (open squares). Cell extracts were subsequently assayed for CAT activity and data plotted as the percentage of the maximal response obtained. Data points represent the mean of assays performed in triplicate. Similar results were obtained in two independent experiments.

upstream of the minimal thymidine kinase promoter and the *CAT* gene [*(CYP3A1)$_2$-tk-CAT*]. Interestingly, in the absence of steroids, PXR.1 was found to have a roughly 20-fold higher basal level of activity than PXR.2 (Figure 5A). Nevertheless, both PXR isoforms were efficiently activated by dexamethasone-t-butylacetate (Figure 5A). Dose–response analysis revealed dexamethasone-t-butylacetate to be a significantly more potent activator of PXR.1 than PXR.2, with EC_{50} values of 0.8 and 5 μM for PXR.1 and PXR.2, respectively (Figures 5B and 5C). Consistent with the results obtained using the GAL4-PXR chimeras, 6,16α-dimethyl pregnenolone, PCN, and RU486 activated full-length PXR.1 on the *CYP3A1* response element but failed to activate full-length PXR.2 (Figures 5B and 5C). 6,16α-dimethyl pregnenolone was the most potent of these compounds, activating PXR.1 with an EC_{50} value of 300 nM (Figure 5B). Based upon these data, we conclude that the full-length PXR isoforms can activate gene expression through the *CYP3A1* DR-3 motif in response to synthetic steroids.

PXRs Are Activated by Naturally Occurring Steroids

As PXR was activated by synthetic steroids, we examined whether a naturally occurring steroid might serve as the endogenous hormone for PXR. Accordingly, CV-1 cells were transfected with expression plasmids for either of the full-length PXR isoforms and the *(CYP3A1)$_2$-tk-CAT* reporter and treated with a variety of natural steroids, including progestins, glucocorticoids, mineralocorticoids, androgens, estrogens, bile acids, and oxysterols. Both PXR.1 and PXR.2 were activated by micromolar concentrations of certain of these steroids. Consistent with our findings that the pregnenolone derivatives 6,16α-dimethyl pregnenolone and PCN activate PXR.1, pregnenolone and its metabolites 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, and 5β-pregnane-3,20-dione activated PXR.1 (Figure 6B). Dose–response analysis revealed that all five of these pregnanes activated PXR.1 with EC_{50} values in the 5–20 μM range (Figure 6C). Since PXR.1 is activated by synthetic glucocorticoids, we were surprised to find that naturally occurring glucocorticoids, including cortisol and corticosterone, had virtually no effect on PXR.1 activity (data not shown).

In analogous cotransfection experiments performed with PXR.2, only 5β-pregnane-3,20-dione was found to induce reporter activity >5-fold (Figure 6B). Notably, pregnenolone, progesterone, and their 17α-hydroxylated derivatives, which were efficacious activators of PXR.1, had little or no activity on PXR.2 (Figure 6B). Thus, while both PXR isoforms are activated by naturally occurring pregnanes, PXR.1 is activated by a broader range of these steroids than PXR.2. The generation of LBD isoforms with distinct activation profiles provides a novel mechanism for increasing the regulatory diversity in the PXR signaling pathway.

Steroids Promote the Interaction of PXR with a Coactivator Protein

We next sought to address whether the steroids that activated PXR did so through direct interactions with the LBD. Due to the lack of radiolabeled derivatives of the more potent PXR activators, we were unable to perform standard binding analyses. However, a number of laboratories have recently demonstrated that ligands

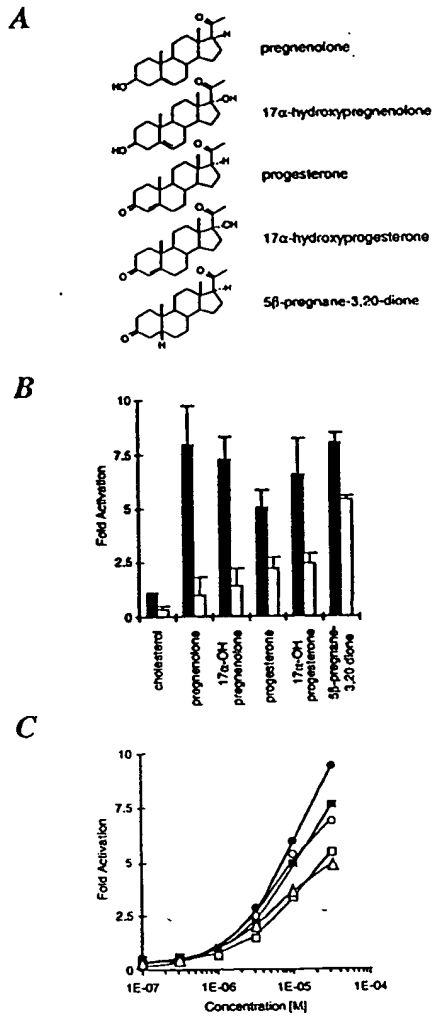

Figure 6. Naturally Occurring Steroids Activate PXR

(A) Structures of naturally occurring steroids that activate PXR.

(B) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 (filled bars) or PXR.2 (open bars) and the *(CYP3A1)$_2$-tk-CAT* reporter plasmid. Transfected cells were treated with 10 μM of the indicated steroids. Cell extracts were subsequently assayed for CAT activity and data plotted as fold-activation relative to cells treated with vehicle (0.1% DMSO) alone. Data points represent the mean of assays performed in triplicate ± SD.

(C) Transfected cells were treated with the indicated concentrations of pregnenolone (closed circles), 17α-hydroxypregnenolone (open circles), progesterone (triangles), 17α-hydroxyprogesterone (open squares), or 5β-pregnane-3,20-dione (closed squares). Cell extracts were subsequently assayed for CAT activity and data plotted as fold-activation relative to cells treated with vehicle (0.1% DMSO) alone. Data points represent the mean of assays performed in triplicate. Similar results were obtained in two independent experiments.

for nuclear receptors induce their interaction with proteins required for optimal transcriptional activation, the so-called coactivator proteins (Horwitz et al., 1996). These ligand-dependent interactions have been exploited as a biochemical assay for demonstrating direct interactions between ligands and their cognate receptors (Krey et al., 1997).

The steroid receptor coactivator protein-1 (SRC-1) has been shown to interact directly with both steroid and nonsteroid nuclear hormone receptors in a ligand-dependent fashion (Onate et al., 1995; Horwitz et al., 1996; Kamei et al., 1996; Takeshita et al., 1996). The interaction of SRC-1 with nuclear receptors is dependent upon the amino acid motif LXXLL found in multiple copies in SRC-1 (Heery et al., 1997; Torchia et al., 1997). In an effort to determine whether the steroid activators of PXR serve as ligands for this orphan receptor, we tested whether a 14 kDa fragment of SRC-1 (SRC-1.14) containing three LXXLL motifs interacted with PXR in a steroid-dependent manner.

SRC-1.14 was expressed in vitro and labeled with [^{35}S]-methionine and [^{35}S]-cysteine, and the LBD of PXR.1 was expressed in *E. coli* as a fusion protein with glutathione-S-transferase (GST). Coprecipitation experiments were performed in the presence of the most potent PXR activators including 6,16α-dimethyl pregnenolone, dexamethasone-t-butylacetate, and PCN. [^{35}S]-SRC-1.14 interacted only weakly with the GST-PXR.1LBD fusion protein in the absence of added compound (Figure 7A). The interaction of [^{35}S]-SRC-1.14 with GST-PXR.1LBD was significantly enhanced in the presence of either dexamethasone-t-butylacetate, 6,16α-dimethyl pregnenolone, or PCN (Figure 7A). Additional experiments performed with 6,16α-dimethyl pregnenolone and PCN revealed that these steroids promoted [^{35}S]-SRC-1.14/PXR.1LBD interactions in a dose-dependent manner (Figure 7B). Consistent with the results of the transfection studies, 6,16α-dimethyl pregnenolone was more potent than PCN in promoting these interactions (Figure 7B). Estradiol did not stimulate interactions between PXR.1 and [^{35}S]-SRC-1.14, indicating that the enhancement was specific for compounds that activate PXR.1 in the transfection assay (Figure 7A). In control experiments, estradiol promoted the efficient interaction of [^{35}S]-SRC-1.14 with a GST-ERαLBD fusion protein (Figure 7A). However, GST-ERαLBD interactions with [^{35}S]-SRC-1.14 were not induced in the presence of PCN, dexamethasone-t-butylacetate, or 6,16α-dimethyl pregnenolone. Taken together, these data provide strong evidence that PCN, dexamethasone-t-butylacetate, and 6,16α-dimethyl pregnenolone serve as ligands for PXR.

Discussion

Identification of a Novel Signaling Pathway for Steroids

It was first observed over 50 years ago that repeated administration of high doses of certain steroids, including progestins and androgens, reduced their own and each others toxic effects. These early observations, together with the later findings that resistance to numerous drugs is sex dependent, that castration of rodents leads to increased drug sensitivity, and that liver homogenates of intact male rats metabolize certain drugs more rapidly than homogenates prepared from castrated animals led to the concept of "catatoxic" steroids; that is, steroids that confer resistance to specific toxins by accelerating their metabolism (reviewed by Kourounakis et al., 1977). It was speculated that catatoxic agents might have utility in the treatment of patients suffering from either drug intoxication or from diseases caused by endogenous substances liable to metabolism (e.g., Cushing's syndrome) (Kourounakis et al., 1977).

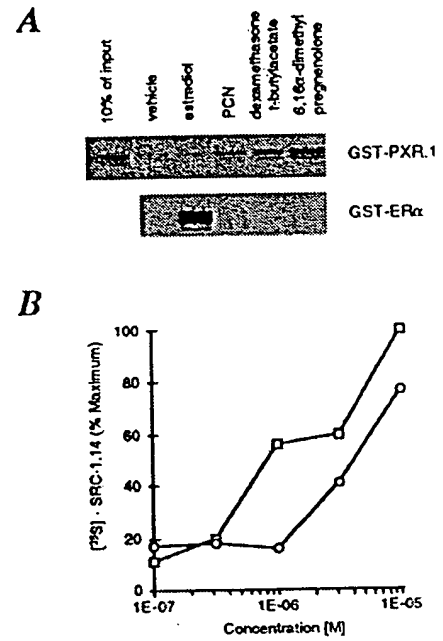

Figure 7. Steroids Induce PXR Interactions with a Fragment of the Coactivator Protein SRC-1

(A) Coprecipitation experiments were performed with bacterially expressed GST-PXR.1LBD (upper panel) or GST-ERαLBD (lower panel) and [^{35}S]-SRC-1.14 synthesized in vitro. [^{35}S]-SRC-1.14 was mixed with either GST-PXR.1LBD or GST-ERαLBD in the presence of vehicle alone (1% DMSO) or 10 μM of estradiol, PCN, dexamethasone-t-butylacetate, or 6,16α-dimethyl pregnenolone as indicated. [^{35}S]-SRC-1.14 complexed with either GST-PXR.1LBD or GST-ERαLBD was precipitated with glutathione-sepharose beads as described in Experimental Procedures. A lane representing 10% of the input [^{35}S]-SRC-1.14 in each reaction is shown on the left. Western blot analysis with an anti-GST antibody revealed that comparable amounts of GST-PXR.1LBD and GST-ERαLBD fusion proteins were used in the assays (data not shown).

(B) Dose-response analysis was performed with [^{35}S]-SRC-1.14 and GST-PXR.1LBD in the presence of the indicated concentrations of 6,16α-dimethyl pregnenolone (squares) or PCN (circles). [^{35}S]-SRC-1.14 was quantitated via scanning densitometry and plotted as a percent of the [^{35}S]-SRC-1.14 precipitated in the presence of 10 μM 6,16α-dimethyl pregnenolone. Data shown represent the average of duplicate points, and similar results were obtained in two separate experiments.

A systematic analysis of steroids in the early 1970s identified the pregnenolone derivative PCN as the most potent catatoxic compound among those tested (Selye, 1971). Insight into the mechanism underlying the catatoxic effects of PCN was provided by the demonstration that this synthetic steroid induces the expression of CYP3A1 and CYP3A2, two closely related members of the P450 family of monooxygenases (Elshourbagy and Guzelian, 1980; Heuman et al., 1982; Hardwick et al., 1983; Schuetz and Guzelian, 1984; Schuetz et al., 1984; Gonzalez et al., 1985). The CYP3A hemoproteins have a remarkably broad substrate specificity, hydroxylating a variety of xenobiotics such as cyclosporin, warfarin, and erythromycin, as well as endogenous steroids including cortisol, progesterone, testosterone, and DHEA-sulfate (Nebert and Gonzalez, 1987; Juchau, 1990). Subsequent studies with the cloned *CYP3A1* gene promoter identified a PCN response element that was highly conserved in the *CYP3A2* gene promoter (Miyata et al., 1995; Quattrochi et al., 1995). This response element was composed of two copies of the nuclear receptor half-site consensus sequence AGTTCA organized as a DR.

In addition to PCN, the expression of *CYP3A1* was also shown to be induced by dexamethasone both in vivo and in cultured hepatocytes (Heuman et al., 1982; Schuetz and Guzelian, 1984; Schuetz et al., 1984). However, the concentrations of dexamethasone required to induce *CYP3A* gene expression were higher than those typically required to activate the classical GR signaling pathway (Schuetz and Guzelian, 1984; Schuetz et al., 1984). Promoter mapping studies showed that dexamethasone induced *CYP3A1* gene expression through the same DR response element as PCN (Quattrochi et al., 1995; Huss et al., 1996). Thus, paradoxically, both high concentrations of dexamethasone, a glucocorticoid, and PCN, an antiglucocorticoid, induced the expression of the *CYP3A1* gene through the same response element.

We now provide several lines of evidence indicating that the orphan nuclear receptor PXR is responsible for mediating the inductive effects of PCN and dexamethasone on *CYP3A* gene expression. First, both dexamethasone and PCN are efficacious activators of the PXR.1 isoform. Second, PXR binds efficiently as a heterodimer with RXR to the conserved DR-3 motifs identified in the *CYP3A1* and *CYP3A2* gene promoters as glucocorticoid and PCN response elements. Finally, we detected PXR expression in only a few tissues, including liver, intestine, and kidney. These are the primary tissues in which the *CYP3A* genes are expressed and induced in response to either dexamethasone or PCN treatment (Heuman et al., 1982; Debri et al., 1995). Our data thus support the existence of a novel signaling pathway for synthetic glucocorticoids and provide a mechanistic explanation for the long-standing paradox as to how both GR agonists and antagonists can exert similar effects on *CYP3A* gene expression. Moreover, the identification of PXR.1 as the PCN receptor provides the tool necessary for the rapid identification of novel pharmacological agents with more potent catatoxic activities.

In addition to inducing *CYP3A* gene expression, PCN is also known to have marked effects on hepatic cholesterol homeostasis in rodents. These effects include significant decreases in the levels of HMG-CoA reductase and cholesterol 7α-hydroxylase gene expression with concomitant reductions in sterol biosynthesis and bile acid secretion (von Bergmann et al., 1975; Mason and Boyd, 1978; Turley and Dietschy, 1984; Stahlberg, 1995). PCN has also been reported to enhance the formation of cholesterol-esters and the hypersecretion of cholesterol into the bile (von Bergmann et al., 1975; Turley and Dietschy, 1984). Thus, PCN affects key aspects of cholesterol metabolism including its biosynthesis, storage, and secretion. Although we cannot exclude the possibility that some of its biological effects might be mediated through the GR or other steroid receptors, it is tempting to speculate that PCN is mimicking the actions of an endogenous hormone that serves to regulate coordinately steroid and sterol metabolism through the activation of PXR in tissues such as liver and intestine. Our data raise the possibility of the existence of regulatory loops through which endogenous PXR hormones feed back to regulate cholesterol homeostasis and feed forward to regulate steroid homeostasis.

What is the natural ligand for PXR? Pregnenolone and progesterone are among the most potent naturally occurring PXR activators that we have identified, activating PXR.1 at low micromolar concentrations. Pregnenolone is one of the most abundant steroids in mammals, circulating in humans and rodents at concentrations that range from roughly 1 to 50 nM (Punjabi et al., 1983; Wichmann et al., 1984; Tietz, 1995). While progesterone levels in human serum are generally in the 1–10 nM range, concentrations can exceed 700 nM during the third trimester of pregnancy (Tietz, 1995). These levels approach those required to activate PXR.1 in vitro. Nevertheless, it remains unclear whether concentrations of progesterone or pregnenolone sufficient to activate PXR.1 are achieved either in serum or in tissues under normal physiological conditions. Our results with PXR may thus be analogous to those obtained with RXR, which was first shown to be activated by micromolar concentrations of all-*trans* retinoic acid (t-RA) prior to the identification of 9-*cis* RA as a high-affinity ligand (Mangelsdorf et al., 1990; Heyman et al., 1992; Levin et al., 1992). Based upon our findings that PXR is activated by pregnenolone and its metabolites and that the synthetic steroid 6,16α-dimethyl pregnenolone activates PXR with an EC_{50} value of 300 nM, we suggest that the natural PXR ligand is likely to be a pregnane.

Perspectives

With the isolation of the androgen receptor in 1988 (Chang et al., 1988; Lubahn et al., 1988), receptors for all of the known nuclear-acting steroid hormones had been cloned. However, studies performed during the past two years with orphan members of the nuclear receptor family have suggested that additional sterols are likely to serve as mammalian hormones. For example, the orphan receptors LXR and SF-1 were recently shown to be activated by physiological concentrations of several oxysterol metabolites of cholesterol (Janowski et al., 1996; Lala et al., 1997; Lehmann et al., 1997). While the biological role of LXR remains less clear, SF-1 is essential for adrenal and gonadal development and regulates the expression of genes required for steroidogenesis (Luo et al., 1994). These data suggested an unexpected hormonal function for oxysterols in the regulation of steroidogenesis.

We have now identified PXR as a novel member of the nuclear receptor family that is efficaciously activated by both natural and synthetic steroids. The activation profile of PXR is distinct from any of the other steroid hormone receptors identified to date, suggesting that this orphan receptor defines a novel endocrine signaling pathway. We conclude that the identification of PXR provides additional evidence for an expanded role for steroid hormones in mammalian physiology and that the elucidation of the biological role of PXR is likely to lead to a better understanding of how steroids elicit their myriad effects.

Experimental Procedures

Chemicals

Dexamethasone-t-butylacetate and RU486 were purchased from Research Plus, Inc. (Bayonne, NJ) and Biomol (Plymouth Meeting, PA), respectively. All other steroids were purchased from either Sigma Chemical Co. (St. Louis, MO) or Steraloids, Inc. (Wilton, NH).

Molecular Cloning of *PXR* cDNAs

Partial mouse sequence for PXR was obtained from an EST deposited in the Washington University/HHMI EST database (accession number AA277370). A 19 bp oligonucleotide derived from this EST sequence (5′ TCTCCCCAGATCGTCCTGG 3′) was used to screen a pCMV-SPORT mouse liver cDNA library (GIBCO-BRL) using Gene Trapper solution hybridization cloning technology (GIBCO-BRL). Five clones were obtained that ranged in size from 1.0 kb to 2.7 kb. Four of these clones encoded PXR.1 and one encoded the DBD and LBD of PXR.2. A clone encoding the full-length PXR.2 isoform-coding region (nucleotides 151 to 1443) was subsequently isolated from mouse liver cDNA through PCR. Sequences were aligned and analyzed by the University of Wisconsin Genetics Computer Group programs.

Plasmids

The expression plasmids pSG5-GAL4-PXR.1LBD and pSG5-GAL4-PXR.2LBD were generated by amplification of cDNA encoding amino acids 105–431 of PXR.1 or 105–390 of PXR.2 by PCR and insertion into a modified pSG5 expression vector (Stratagene) containing DNA encoding the DBD of GAL4 (amino acids 1–147) and the SV40 Tag nuclear localization signal (APKKKRKVG) inserted upstream of a multiple cloning site. The *$(UAS)_5$-tk-CAT* reporter plasmid has been previously described (Lehmann et al., 1995). The expression vectors pSG5-PXR.1 and pSG5-PXR.2 were generated by amplification of cDNA encoding amino acids 1–431 of PXR.1 or 1–390 of PXR.2 and insertion into pSG5. The reporter plasmid *$(CYP3A1)_2$-tk-CAT* was generated by insertion of two copies of a double-stranded oligonucleotide containing the *CYP3A1* DR-3 RE (5′ GATCAGACAGTTCATGAAGTTCATCTAGATC 3′) (Quattrochi et al., 1995; Huss et al., 1996) into the BamHI site of pBLCAT2 (Luckow and Schütz, 1987). The bacterial expression vector pGEX-PXR.1LBD was generated by PCR amplification of cDNA encoding amino acids 105–431 of PXR.1 and insertion into a pGEX-2T vector (Pharmacia) that had been modified to contain KpnI and NotI restriction sites. The bacterial expression vector pGEX-ERαLBD was generated by PCR amplification of cDNA encoding amino acids 251–595 of human ERα (Green et al., 1986) and insertion into pGEX-2T. The expression plasmid for the SRC-1.14 fragment was generated by PCR amplification of DNA encoding amino acids 632–754 of human SRC-1 (Takeshita et al., 1996) and insertion into the expression vector pRSETC (Invitrogen). All constructs were confirmed by sequence analysis.

Cotransfection Assays

CV-1 cells were plated in 24-well plates in DME medium supplemented with 10% charcoal-stripped fetal calf serum at a density of 1.2×10^5 cells per well. In general, transfection mixes contained 33 ng of receptor expression vector, 100 ng of reporter plasmid, 200 ng of β-galactosidase expression vector (pCH110, Pharmacia), and 166 ng of carrier plasmid. Cells were transfected overnight by lipofection using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. The medium was changed to DME medium supplemented with 10% delipidated calf serum (Sigma) and cells were incubated for an additional 24 hr. Cell extracts were prepared and assayed for CAT and β-galactosidase activities as described previously (Lehmann et al., 1995).

Northern Analysis

An approximately 1.0 kb fragment encoding the LBD of PXR.1 (nucleotides 463–1446) was ^{32}P-labeled by random priming using the T7 Quick-Prime kit (Pharmacia). This radiolabeled fragment was used to probe mouse multiple-tissue Northern blots (OriGene, Rockville, MD). Blots were hybridized in ExpressHyb solution (Clontech, Palo Alto, CA) at 65°C for 18 hr. Final washes were performed with 0.1× SSC, 0.1% SDS at 65°C.

In Situ Hybridization Analysis

Embryonic day 18 (E18) mice (C57bl/CBA and NMRI, Bomholt Breeding and Research Center, Copenhagen, Denmark) were used in the studies. Tissues were sectioned at 14 μm and thaw-mounted

onto slides (ProbeOn, Fischer). Three different oligonucleotide probes (Pharmacia Biotech, Sollentuna, Sweden) designed to hybridize to *PXR* mRNA were used. Oligonucleotides were radiolabeled using [^{35}S]-deoxyadenosine 5'-α-thio-triphosphate (NEN) at the 3' end using terminal deoxynucleotidyl transferase (Amersham). Labeled oligonucleotides were hybridized to tissue sections and mRNA expression was first detected by phosphoimaging (BASF 3000 Phosphoimager, Fuji) followed by film emulsion autoradiography (Dagerlind et al., 1992). Sections were examined using light- and dark-field microscopy (Axiophot, Zeiss) and photographed (Ektachrome 64T, Kodak). Positives were scanned (SprintScan 35, Polaroid) and processed using Photoshop and PageMaker, Adobe. The following oligonucleotide sequences were used as probes: probe 1 (PXR.1 and PXR.2 specific), 5' GGAGCCTCAATCTTTTCCC TCTTCTTCCTCTTGATCAAGGCCCGC 3'; probe 2 (PXR.1 specific), 5' CTTCCAACAGTGAGGCCTGCAGAAACTCTGGAAGCTCACAGC CAC 3'; probe 3 (PXR.1 and PXR.2 specific), 5' TGGGCTCTTCCAAG GCAGAGTCTGCTTCT TCACACTGTACAAGGCC 3'. A 50 bp random oligonucleotide was used as a negative control.

Gel Mobility Shift Assays

PXR.1, PXR.2, and RXRα were synthesized in vitro using the TNT rabbit reticulocyte lysate coupled in vitro transcription/translation system (Promega) according to the manufacturer's instructions. Gel mobility shift assays (20 μl) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM DTT, 0.2 μg of poly(dI-dC), and 2.5 μl each of in vitro–synthesized PXR and RXR proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μl) through the addition of unprogrammed lysate. Competitor oligonucleotides were included at a 5-fold or 25-fold excess as indicated in the figure legends. After a 10 min incubation on ice, 10 ng of ^{32}P-labeled oligonucleotide was added and the incubation continued for an additional 10 min. DNA–protein complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE (1× TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°C. The following double-stranded oligonucleotides were used as either radiolabeled probes or competitors: *CYP3A1*, 5' GATGCAGACAGTTCATGAAGTTCATCT AGATC 3' (Quattrochi et al., 1995); Δ*CYP3A1*, 5' GATCAGACAGAAC ATGAAGAACATCTAGATC 3'; *CYP3A2*, 5' GATCAAACAGTTCATAA AGTTCATCTAGATC 3' (Miyata et al., 1995).

SRC-1.14 Coprecipitation Assay

GST-PXR.1LBD and GST-ERαLBD fusion proteins were expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in protein lysis buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM DTT, and 1% NP-40 followed by centrifugation at 40,000 × g for 30 min. Glycerol was added to the resulting supernatant to a final concentration of 10%. Lysates were stored at −80°C. [^{35}S]-SRC-1.14 was generated using the TNT rabbit reticulocyte system (Promega) in the presence of Pro-Mix (Amersham). Coprecipitation reactions included 25 μl of lysate containing GST-PXR.1LBD or GST-ERαLBD fusion proteins or control GST; 25 μl incubation buffer (50 mM KCl, 40 mM HEPES [pH 7.5]; 5 mM β-mercaptoethanol; 1% Tween-20, 1% non-fat dry milk); 5 μl [^{35}S]-SRC-1.14; and either PCN; dexamethasone-t-butylacetate; 6,16α-dimethyl pregnenolone; estradiol; or control DMSO. The mixtures were incubated for 25 min at 4°C with gentle mixing prior to the addition of 15 μl of glutathione-sepharose 4B beads (Pharmacia) that had been extensively washed with protein lysis buffer. Reactions were incubated with gentle mixing at 4°C for an additional 20 min. The beads were pelleted at 2000 rpm in a microfuge and washed 3 times with protein lysis buffer containing either PCN; dexamethasone-t-butylacetate; 6,16α-dimethyl pregnenolone; estradiol; or control DMSO. After the last wash, the beads were resuspended in 25 μl of 2× SDS-PAGE sample buffer containing 1 mM DTT. Samples were heated at 100°C for 5 min and loaded onto a 10% Bis-Tris PAGE gel. Autoradiography was performed for 2–4 days. Assays were quantitated using a Molecular Dynamics Computing Densitometer and Image Quant software.

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GenBank Accession Number

The PXR sequence has been deposited in GenBank (accession number AF031814).

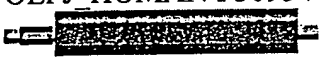
In re: Glucksmann *et al.*
Appl. No. 09/464,685
Filed: December 16, 1999

APPENDIX E

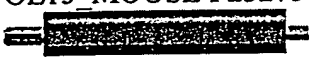
Domain structure of proteins in the 7tm_1 Seed alignment

Pfam domains are large boxes. Small three-colored boxes are Pfam-B clusters. Mouseover to see domain descriptions. Click on box to enter family page. (Javascript is used for mouseover functionality.)

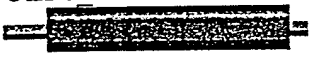
OLFJ_HUMAN P30954 OLFACTORY RECEPTOR-LIKE PROTEIN HGMP07J.



OL15_MOUSE P23275 OLFACTORY RECEPTOR 15 (OR3).

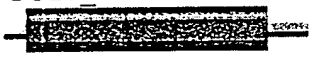


OLF6_RAT P23267 OLFACTORY RECEPTOR-LIKE PROTEIN F6.



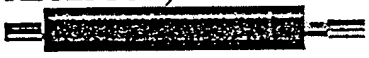
OLF1_CHICK P37067 OLFACTORY RECEPTOR-LIKE PROTEIN COR1.

GU27_RAT P34987 GUSTATORY RECEPTOR GUST27.

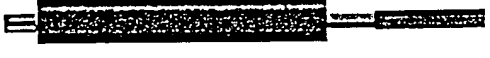


RTA_RAT P23749 PROBABLE G PROTEIN-COUPLED RECEPTOR RTA.

TA2R_HUMAN P21731 THROMBOXANE A2 RECEPTOR (TXA2-R) (PROSTANOID TP RECEPTOR).



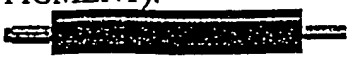
PE24_HUMAN P35408 PROSTAGLANDIN E2 RECEPTOR, EP4 SUBTYPE (PROSTANOID EP4 RECEPTOR) (PGERECEPTOR, EP4 SUBTYPE).



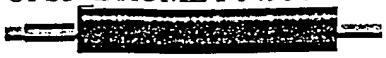
UL33_HCMVA P16849 G-PROTEIN COUPLED RECEPTOR HOMOLOG UL33.

OPSB_HUMAN P03999 BLUE-SENSITIVE OPSIN (BLUE CONE PHOTORECEPTOR

PIGMENT).

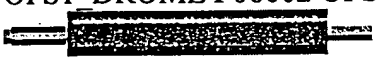


OPS3_DROME P04950 OPSIN RH3 (INNER R7 PHOTORECEPTOR CELLS OPSIN).

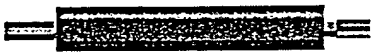


OPSD_LOLFO P24603 RHODOPSIN.

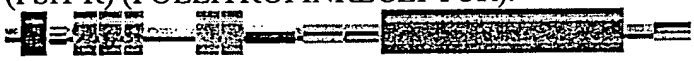
OPS1_DROME P06002 OPSIN RH1 (OUTER R1-R6 PHOTORECEPTOR CELLS OPSIN).



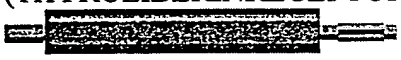
V2R_HUMAN P30518 VASOPRESSIN V2 RECEPTOR (RENAL-TYPE ARGININE VASOPRESSIN RECEPTOR)(ANTIDIURETIC HORMONE RECEPTOR) (AVPR V2).



FSHR_BOVIN P35376 FOLLICLE STIMULATING HORMONE RECEPTOR PRECURSOR (FSH-R) (FOLLITROPINRECEPTOR).

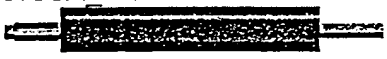


TRFR_HUMAN P34981 THYROTROPIN-RELEASING HORMONE RECEPTOR (TRH-R) (THYROLIBERINRECEPTOR).

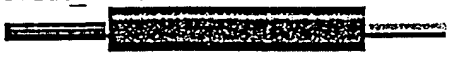


NTR1_HUMAN P30989 NEUROTENSIN RECEPTOR TYPE 1 (NT-R-1) (HIGH-AFFINITY LEVOCABASTINE-INSENSITIVE NEUROTENSIN RECEPTOR) (NTRH).

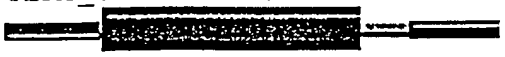
NY1R_HUMAN P25929 NEUROPEPTIDE Y RECEPTOR TYPE 1 (NPY1-R).



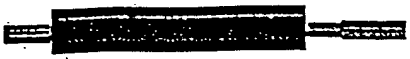
NYR_DROME P25931 NEUROPEPTIDE Y RECEPTOR (NPY-R) (PR4 RECEPTOR).



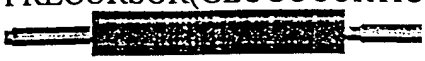
TLR1_DROME P30974 TACHYKININ-LIKE PEPTIDES RECEPTOR 86C (NKD).



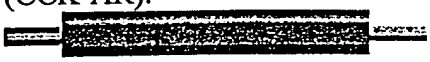
NK1R_CAVPO P30547 SUBSTANCE-P RECEPTOR (SPR) (NK-1 RECEPTOR) (NK-1R).



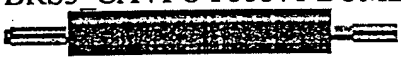
GCRC_MOUSE P30731 PROBABLE G PROTEIN-COUPLED RECEPTOR FROM T-CELLS PRECURSOR(GLUCOCORTICOID-INDUCED RECEPTOR).



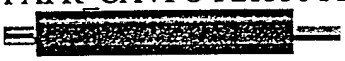
CCKR_HUMAN P32238 CHOLECYSTOKININ TYPE A RECEPTOR (CCK-A RECEPTOR) (CCK-AR).



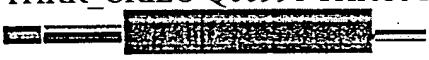
BRS3_CAVPO P35371 BOMBESIN RECEPTOR SUBTYPE-3 (BRS-3).



PAFR_CAVPO P21556 PLATELET ACTIVATING FACTOR RECEPTOR (PAF-R).



THRR_CRILO Q00991 THROMBIN RECEPTOR PRECURSOR.

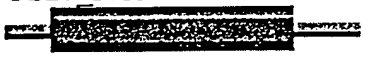


P2Y5_CHICK P32250 P2Y PURINOCEPTOR 5 (P2Y5) (PURINERGIC RECEPTOR 5) (6H1).

EBI2_HUMAN P32249 EBV-INDUCED G PROTEIN-COUPLED RECEPTOR 2 (EBI2).

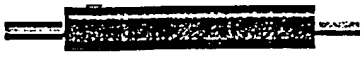
US28_HCMVA P09704 G-PROTEIN COUPLED RECEPTOR HOMOLOG US28 (HHRF3).

US27_HCMVA P09703 G-PROTEIN COUPLED RECEPTOR HOMOLOG US27 (HHRF2).

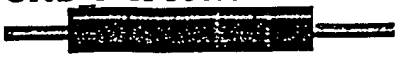


C5AR_CANFA P30992 C5A ANAPHYLATOXIN CHEMOTACTIC RECEPTOR (C5A-R).

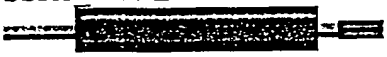
RDC1_CANFA P11613 G PROTEIN-COUPLED RECEPTOR RDC1.



G10D_RAT P31392 PROBABLE G PROTEIN-COUPLED RECEPTOR G10D (NOW).



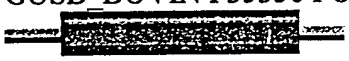
SSR1_HUMAN P30872 SOMATOSTATIN RECEPTOR TYPE 1 (SS1R) (SRIF-2).



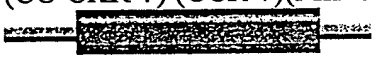
OPRD_MOUSE P32300 DELTA-TYPE OPIOID RECEPTOR (DOR-1) (K56) (MSL-2).

APJ_HUMAN P35414 PROBABLE G PROTEIN-COUPLED RECEPTOR APJ.

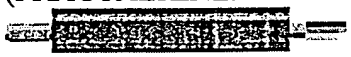
GUSB_BOVIN P35350 POSSIBLE GUSTATORY RECEPTOR TYPE B (PPR1 PROTEIN).



CKR7_HUMAN P32248 C-C CHEMOKINE RECEPTOR TYPE 7 PRECURSOR (C-C CKR-7) (CC-CKR-7) (CCR-7)(MIP-3 BETA RECEPTOR) (EBV-INDUCED G PROTEIN-COUPL

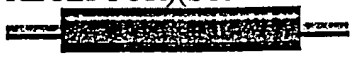


C3X1_RAT P35411 CX3C CHEMOKINE RECEPTOR 1 (C-X3-C CKR-1) (CX3CR1) (FRACTALKINERECEPTOR) (GPR13) (RBS11).

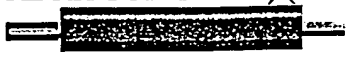


CKR1_HUMAN P32246 C-C CHEMOKINE RECEPTOR TYPE 1 (C-C CKR-1) (CC-CKR-1) (CCR-1) (CCR1)(MACROPHAGE INFLAMMATORY PROTEIN-1 ALPHA RECEPTOR) (M

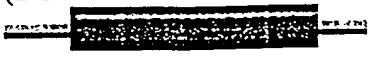
CCR4_BOVIN P25930 C-X-C CHEMOKINE RECEPTOR TYPE 4 (CXC-R4) (CXCR-4) (SDF-1 RECEPTOR)(STROMAL CELL-DERIVED FACTOR 1 RECEPTOR) (FUSIN) (LEUK



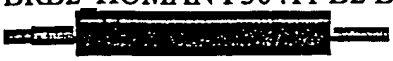
IL8A_HUMAN P25024 HIGH AFFINITY INTERLEUKIN-8 RECEPTOR A (IL-8R A) (IL-8 RECEPTOR TYPE1) (CXCR-1) (CDW128).



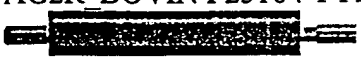
CCR5_HUMAN P32302 C-X-C CHEMOKINE RECEPTOR TYPE 5 (CXC-R5) (CXCR-5) (BURKITT'S LYMPHOMARECEPTOR 1) (MONOCYTE-DERIVED RECEPTOR 15) (MDR15).



BRB2_HUMAN P30411 B2 BRADYKININ RECEPTOR (BK-2 RECEPTOR).



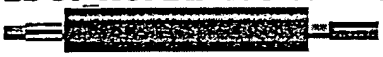
AG2R_BOVIN P25104 TYPE-1 ANGIOTENSIN II RECEPTOR (AT1).



AG22_MOUSE P35374 TYPE-2 ANGIOTENSIN II RECEPTOR (AT2).

MC3R_MOUSE P33033 MELANOCORTIN-3 RECEPTOR (MC3-R).

EDG1_HUMAN P21453 PROBABLE G PROTEIN-COUPLED RECEPTOR EDG-1.

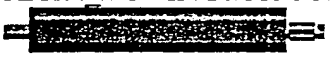


CB2R_HUMAN P34972 CANNABINOID RECEPTOR 2 (CB2) (CB-2) (CX5).

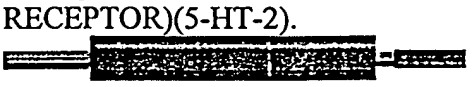
CB1R_HUMAN P21554 CANNABINOID RECEPTOR 1 (CB1) (CB-R) (CANN6).

ACM1_HUMAN P11229 MUSCARINIC ACETYLCHOLINE RECEPTOR M1.

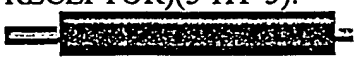
AA1R_BOVIN P28190 ADENOSINE A1 RECEPTOR.



5H2A_CRIGR P18599 5-HYDROXYTRYPTAMINE 2A RECEPTOR (5-HT-2A) (SEROTONIN RECEPTOR)(5-HT-2).

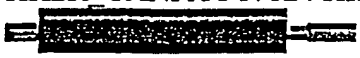


5H5A_MOUSE P30966 5-HYDROXYTRYPTAMINE 5A RECEPTOR (5-HT-5A) (SEROTONIN RECEPTOR)(5-HT-5).

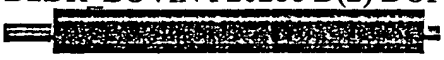


5H6_RAT P31388 5-HYDROXYTRYPTAMINE 6 RECEPTOR (5-HT-6) (SEROTONIN RECEPTOR)(ST-B17).

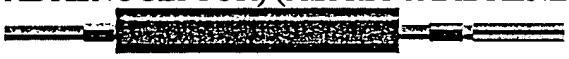
HH2R_CANFA P17124 HISTAMINE H2 RECEPTOR (GASTRIC RECEPTOR I).



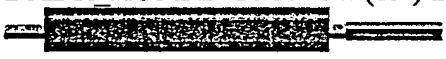
D2DR_BOVIN P20288 D(2) DOPAMINE RECEPTOR.



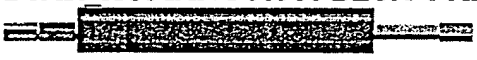
A1AD_HUMAN P25100 ALPHA-1D ADRENERGIC RECEPTOR (ALPHA 1D-ADRENOCEPTOR) (ALPHA-1AADRENERGIC RECEPTOR).



DADR_HUMAN P21728 D(1A) DOPAMINE RECEPTOR.



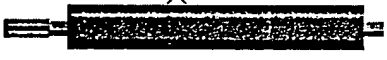
B1AR_HUMAN P08588 BETA-1 ADRENERGIC RECEPTOR.



5HT1_DROME P20905 5-HYDROXYTRYPTAMINE RECEPTOR 1 (5-HT RECEPTOR) (SEROTONIN RECEPTOR).

5H7_HUMAN P34969 5-HYDROXYTRYPTAMINE 7 RECEPTOR (5-HT-7) (5-HT-X) (SEROTONIN RECEPTOR)(5HT7).

5H1B_HUMAN P28222 5-HYDROXYTRYPTAMINE 1B RECEPTOR (5-HT-1B) (SEROTONIN RECEPTOR)(5-HT-1D-BETA) (S12).



5H1A_HUMAN P08908 5-HYDROXYTRYPTAMINE 1A RECEPTOR (5-HT-1A) (SEROTONIN RECEPTOR) (5-HT1A) (G-21).